Biochemical analysis of the α-terpineol/1,8-cineole cyclization reaction of the cineole synthase of *Nicotiana suaveolens*.

Dissertation

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II. List of figures

III. List of tables

IV. List of abbreviations

%	percent
°C	degree Celsius
&	and
π	pi
α	(gr. alpha)
β	(gr. beta)
γ	(gr. gamma)
Å	angstrom
3D	three dimensional
^{3}H	tiritum labeled hydrogen
µl	microliter
Aqua dest.	distilled water
C_5	five carbons
C1	carbon atom one
CDP	Disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2′-(5-chlorotricyclo[3.3.1.13.7]decan])-4-yl]-1-phenyl phosphate
Co^{2+}	cobalt ion
CoA	Coenzyme A
DMAPP	dimethylallyl pyrophosphate
DNA	desoxyribonucleic acid
Dpn	*Diplococcus pneumoniae*
DOXP	1-desoxy-D-xylulose-5-phosphate
DTT	dithiothreitol
(*E*)	trans isomerism
E. coli	*Escherichia coli*
e. g.	exempli gratia (lat. for example)
et al.	et alia (lat. and others)
FPP	farnesyl pyrophosphate
ft	feet (1 meters = 3.2808 ft)
kDa	kilodalton
K_M	Michaelis-Menten constant

v_{max}	maximum velocity
g	gram
GC-MS	gas chromatography-mass spectrometer
GDP	geranyl diphosphate
GGPP	geranylgeranyl pyrophosphate
GFPP	geranylfarnesol pyrosphosphate
GPP	geranyl pyrophosphate
gr.	greek
h	hour
H^+	proton
H_2O	water
IPP	isopentenyl pyrophosphate
IPTG	isopropyl-β-$_D$-thiogalactopyranoside
k_{cat}	turnover number
kPa	kilopascal
lat.	latin
LB	lysogene broth
LDP	linalyl diphosphate
M	molar
m	meter
mCi	milli Curie
MEP	2-methyl-D-erythritol-4-phosphate
mg	milligram
$MgCl_2$	magnesium dichloride
min	minute
mL	milliliter
mm	millimeter
$MnCl_2$	manganese dichloride
m/z	mass-to-charge ratio
N.	*Nicotiana*
n	number of experiments
$n_{biol.}$	number of biological replicates
NaCl	sodium chloride
ng	nanogram

Ni^{2+}-NTA	Nickel-Nitrilotriessigsäure
nM	Nano molar
OD_{600}	optical density by 600 nm wave length
OPP	ortho pyrophosphate
PCR	polymerase chain reaction
pH	*potentia Hydrogenii*
pkat	picokatal
PVDF	polyvinyldifluoride
rpm	rounds per minute
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
sec.	second
Tpsb	terpene synthase subfamily b
TRIS	Tris-aminomethane
V	volt
VOC	volatile organic compound
w/v	weight/volume

Abbreviations of the amino acids

A, ala	alanine
C, cys	cysteine
D, asp	aspartate
E, glu	glutamate
F, phe	phenylalanine
G, gly	glycine
H, his	histidine
I, ile	isoleucine
L, leu	leucine
M, met	methionine
N, asn	asparagine
R, arg	arginine
S, ser	serine
T, thr	threonine

V, val	valine
W, trp	tryptophan
X	unidentified amino acid
Y, tyr	tyrosine

1. Introduction

1.1 Synthesis and function of terpenes

Plants synthesize a broad range of secondary metabolites with different functions. Terpenes and terpenoids are one of the largest classes of secondary metabolites and are produced by diverse species of plants, algae, fungi, bacteria and also archaea (Lichtenthaler 1999, Rynkiewicz et al. 2001, Aaron & Christianson 2010, Liu et al. 2014). Terpenes are naturally occurring hydrocarbons derived from isoprene units (C5 bodies); terpenoids are further oxygenated or contain functional groups (e.g., hydroxyl groups). Terpenes are classified as hemi-, mono-, sesqui-, di-, sester-, tri- and tetraterpenes based on the number of isopentenyl pyrophosphate (IPP) units (Figure 1). Hemiterpenes are derived from isopentenyl pyrophosphate, whereas monoterpenes are derived from geranyl pyrophosphate (GPP). The addition of an IPP unit to GPP forms the sesquiterpene precursor farnesyl pyrophosphate (FPP). Subsequent addition of IPP units to FPP and geranylgeranyl pyrophosphate (GGPP) generates the diterpene and sesterpene precursors GGPP and geranylfarnesol pyrophosphate (GFPP), respectively. Triterpenes and tetraterpenes are formed from two farnesyl pyrophosphates or two geranylgeranyl pyrophosphates, respectively.

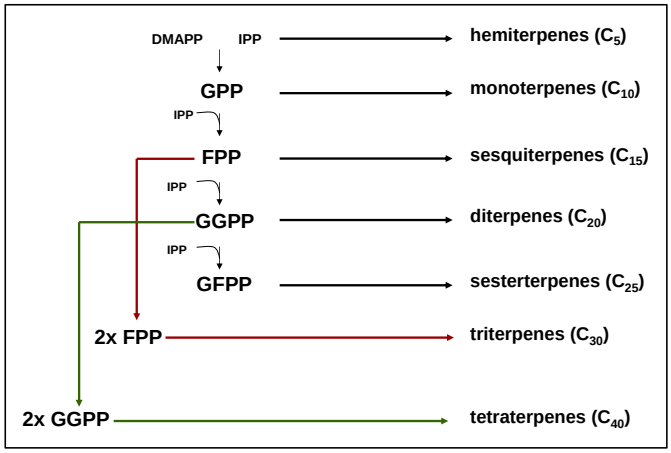


Figure 1: Formation of different terpenes. The precursor of hemiterpenes is isopentenyl pyrophosphate (IPP). Geranyl pyrophosphate (GPP) formed by addition of IPP and dimethylallyl pyrophosphate (DMAPP) is the precursor of monoterpenes. The addition of IPP units yields the precursors for other terpenes. Farnesyl pyrophosphate (FPP) serves as precursor for sesquiterpenes, geranylgeranyl pyrophosphate (GGPP) for diterpenes, and geranylfarnesol pyrophosphate (GFPP) for sesterterpenes. Triterpenes derive from two units FPP and tetraterpenes from two units of GGPP. (Dewick 2009, modified).

The isoprene rule (Wallach 1887; Ruzicka 1953) dictates that the IPP units are linked by a head-to-tail reaction (Figure 2). The isopropyl unit is the head and the end of the isoprene unit is termed tail. The formation of geranyl

pyrophosphate follows the head-to-tail rule. The head of the IPP is linked with the tail of DMAPP. The following reaction of the GPP by a head-to-tail reaction to an IPP unit resulted in farnesyl diphosphate, the precursor of sesquiterpenes. There are also two other mechanisms known: head-to-head or tail-to-tail reaction (Figure 2). The head-to-head linkage of two farnesyl diphosphates results in squalene, a triterpene.

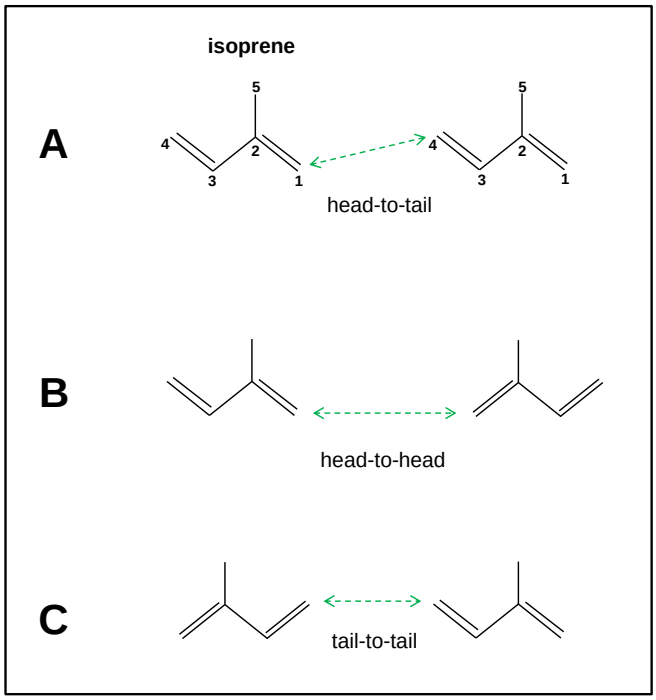


Figure 2: The isoprene rule and additional linkage possibilities. A: Head-to-tail reaction of two isoprene units. Linkage between the C1 of the head of isoprene and the C4 of the tail of a second isoprene. (isoprene rule) **B**: Head-to-head linkage between the C1 of the head and C1 of another head. **C**: Tail-to-tail linkage between two C4 atoms. (Gamini Gunawardena, modified).

Terpenes and terpenoids are further divided into cyclic and acyclic compounds. The monoterpenes limonene, 1,8-cineole or sabinene are examples of cyclic compounds, whereas (*E*)-β-ocimene or β-myrcene are acyclic. Monoterpenes are classified as volatile organic compounds (VOCs), which are characterized by a lipophilic character and molecular masses of less than 300 Dalton (Dudareva et al. 2004). Monoterpenes are often a major part of the floral scent bouquet of seed plants (Knudsen 2006). They are emitted by different plant organs, e.g., flowers, leaves, and roots, and released to the atmosphere or, in the case of roots, the rhizosphere (Chen et al. 2004). These VOCs are detected by various organisms (e.g., insects) and may have different functions for plants. Released monoterpenoid plant volatiles serve as attractants and guides for pollinators (Reinhard et al. 2004; Dudareva et al. 2004), as a defense mechanism to protect the plant against herbivores and abiotic stresses

(Pichersky & Gershenzon 2002), and as intra- and interspecific plant communication molecules (Llusià et al. 1996). Takabayashi et al. (1994) provided evidence that plants produce volatiles as allelochemicals upon damage by herbivores. These volatiles attract natural enemies against these herbivores. Damage of *Gossypium hirsutum* by insects results in the emission of volatile compounds to attract their predator, *Cariochiles nigiceps* (Paré & Tumlinson 1997). Arimura et al. (2004) demonstrated that synthesis of the volatile compound (*E*)-β-ocimene is up-regulated in *Lotus japonicus* to protect the plant against pathogens. In addition to protection and defense of plant habitat, the emission of volatile compounds attracts pollinators to preserve the plant's life cycle. Hawkmoth-pollinated plants, e.g., *Clarkia breweri*, emit linalool to attract these nocturnal insects (Pichersky et al. 1994).

1.1.1 The biochemical reaction mechanism of monoterpenes

Biosynthesis of isoprene

The precursor of all terpenoids is IPP, which is also known as "activated isoprene" (Ruzicka 1953). IPP is synthesized via two different pathways. One pathway is the acetate-mevalonate pathway (Qureshi & Porter, 1981). This pathway occurs in the cytosol and requires three activated units of acetic acid (Acetyl-CoA). First, two units of acetyl-CoA react to form acetoacetyl-CoA (Figure 3), followed by the addition of a third unit of acetyl-CoA the hydroxyl-methylglutaryl-CoA synthase synthesizes 3-hydroxy-3-methylglutaryl CoA. The elimination of CoA by hydroxy-methylglutaryl reductase produces mevalonic acid. Mevalonic acid is phosphorylated and decarboxylated twice by two kinases to form IPP. The isomerization of IPP produces dimethylallylpyrophosphate (DMAPP).

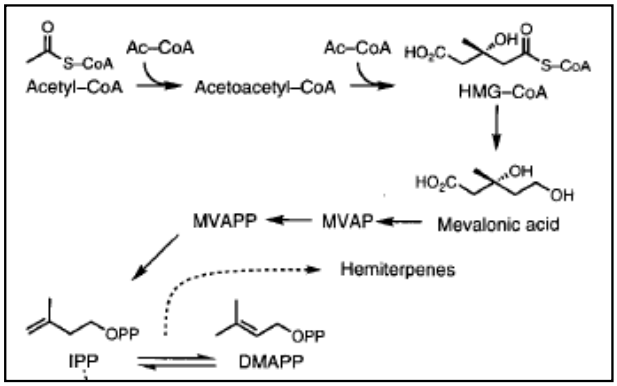


Figure 3: Acetate-mevalonate pathway. Synthesis of IPP via mevalonic acid in the cytosol compartment. Ac-CoA: Acetyl-CoA (activated acetic acid), HMG-CoA: Hydroxy-methylglutaryl-CoA, MVAP: mevalonic acid phosphate, MVAPP: mevalonic acid diphosphate, IPP: Isopentenylpyrophosphate, DMAPP: Dimethylallylpyrophosphate. (McGarvey & Croteau 1995, modified)

Sesqui-, tri- and polyterpenes are synthesized via the acetate-mevalonate pathway. This pathway has been detected in higher plants, algae, bacteria, archaea, and fungi and was first described in human tissue (Eisenreich et al. 2001).

Higher plants and bacteria also synthesize IPP via a mevalonate-independent pathway called the DOXP (1-desoxy-D-xylulose-5-phosphate) or MEP (2-methyl-D-erythritol-4-phosphate) pathway (Rohmer et al. 1993; Lichtenthaler 1999). This pathway occurs only in chloroplasts in plants. The existence of this pathway in bacteria supports the hypothesis that chloroplasts are of prokaryotic origin. The MEP pathway begins with the precursors pyruvate and glyceraldehyde-3-phosphate, which form 1-deoxy-D-xylulose-5-phosphate via the action of DOXP-synthase (Figure 4). Isomerization and reduction by the 1-deoxy-D-xylulose-5-phosphate reductoisomerase leads to the intermediate 2-C-methyl-D-erythritol-4-phospahte. 2-C-Methylerythtritol-2,4-cyclodiphosphate is formed via the enzyme 2-C-methylerythritol 4-phosphate cytidyl transferase and phosphorylation by 4-(cytidine 5´-diphospho)-2-C-methylerythritol kinase. Subsequent dehydration in the last step of the pathway generates IPP. Hemi-, mono- and diterpenes are formed by the MEP pathway. For example, cineole is synthesized by *Eucalyptus globules* (Rieder et al. 2000) via this pathway.

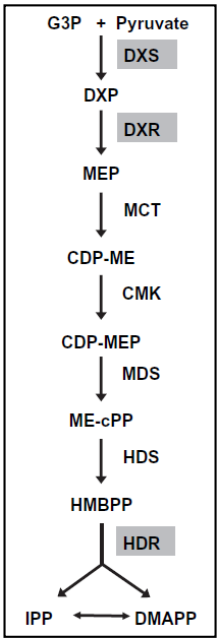


Figure 4: MEP (2-methyl-D-erythritol-4-phosphate) pathway. Abbreviation of the intermediates are G3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; MEP: 2-C-methyl-D-erythritol 4-phosphate; CDP-ME: 4-(cytidine 5´-diphospho)-2-C-methyl-D-erythritol; CDP-MEP: 2-phospho-4-(cytidine 5´-di-phospho)-2-C-methyl-D-erythritol; ME-cPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate. Abbreviation of the enzymes are DXS: DXP synthase; DXR: DXP reductoisomerase; MCT: CDP-ME synthase; CMK: CDP-MEkinase; MDS: ME-cPP synthase; HDS, HMBPP synthase: HDR, HMBPP reductase; IDI: IPP isomerase; GPP: geranyl diphosphate; GGPP: geranylgeranyl diphosphate; ABA, abscisic acid (Cordoba et al. 2009, modified)

The acetate-mevalonate pathway and MEP-pathway function together to synthesize IPP (Dubey et al. 2003). Studies of *Matricaria recutita* and *Gingko biloba* (Adam & Zapp 1998; Schwarz 1994) have demonstrated that these two pathways cooperate to synthesize isoprenoid units for sesquiterpene and diterpene synthesis, respectively (Dubey et al. 2003).

From IPP to monoterpenes

This study focus on the synthesis of the so called “cineole cassette” monoterpenes. This “cineole cassette” includes α-pinene, β-pinene, sabinene, β-myrcene, limonene, 1,8-cineole and α-trepioneol (Raguso et al. 2006).

The reaction mechanism for the synthesis of the “cineole cassette” monoterpenes and monoterpenoids begins with the synthesis of the precursor for all terpene skeletons: IPP. The addition of a second unit of IPP leads to geranyl pyrophosphate (GPP). GPP is the substrate for monoterpene synthases, which catalyze the formation of monoterpenes. Figure 5 shows the reaction pathways of monoterpenoids, which feature several carbocationic intermediates in various reactions (dehydration, ring closure, hydration) in monoterpene synthesis (Croteau et al. 1989; Degenhardt et al. 2009). GPP is first ionized to the geranyl cation by the elimination of the pyrophosphate group.

The conversion of the geranyl cation via the addition of a pyrophosphate group results in linalyl diphosphate (LDP), first in the transoid conformation and followed by the cisoid conformation (isomerization). The following ionization to the linalyl cation is essential to facilitate cyclization to a ring (Degenhardt et al. 2009). The acyclic structure of the linalyl cation is closed by attack of C1 on the double bond of C6 and results in the α-terpinyl cation, which is the carbocationic intermediate for all cyclic monoterpenes. Nucleophilic attack of a water molecule on the C7 atom of the α-terpinyl cation yields α-terpineol (Croteau et al. 1994). The deprotonation of the α-terpinyl cation results in the formation of limonene (Rajaonarivony et al. 1992). α-Pinene and β-pinene are synthesized via the pinyl cation and deprotonation. Sabinene is synthesized via two additional carbocation intermediates. First, a hydride shift leads to the terpinene-4-yl cation, which will undergo intramolecular ring closure between the C2-C6 atoms to generate the thujyl cation (Wise et al. 1999; Crowell et al. 2002; Dewick 2002; Degenhardt et al. 2009). The deprotonation of the thujyl cation leads to sabinene. β-Myrcene is an acyclic monoterpene that can be synthesized via the geranyl cation or linalyl cation by deprotonation (Dudareva et al. 2003; Degenhardt et al. 2009).

The synthesis of 1,8-cineole is reported via α-terpineol (Croteau et al. 1994; Chen et al. 2004; Degenhardt et al. 2009) but the mechanism to 1,8-cineole is not exactly enlightened. It is known that the double bond of α-terpineol is protonated and this results in an intermediate which is the precursor for the cyclization to 1,8-cineole (Wheeler & Croteau 1987, Croteau et al. 1994, Wise et al. 1998, Wise et al. 2002). This mechanism is not fully understood and questionable. Possibly, there occurs a reaction pathway independent from α-terpineol.

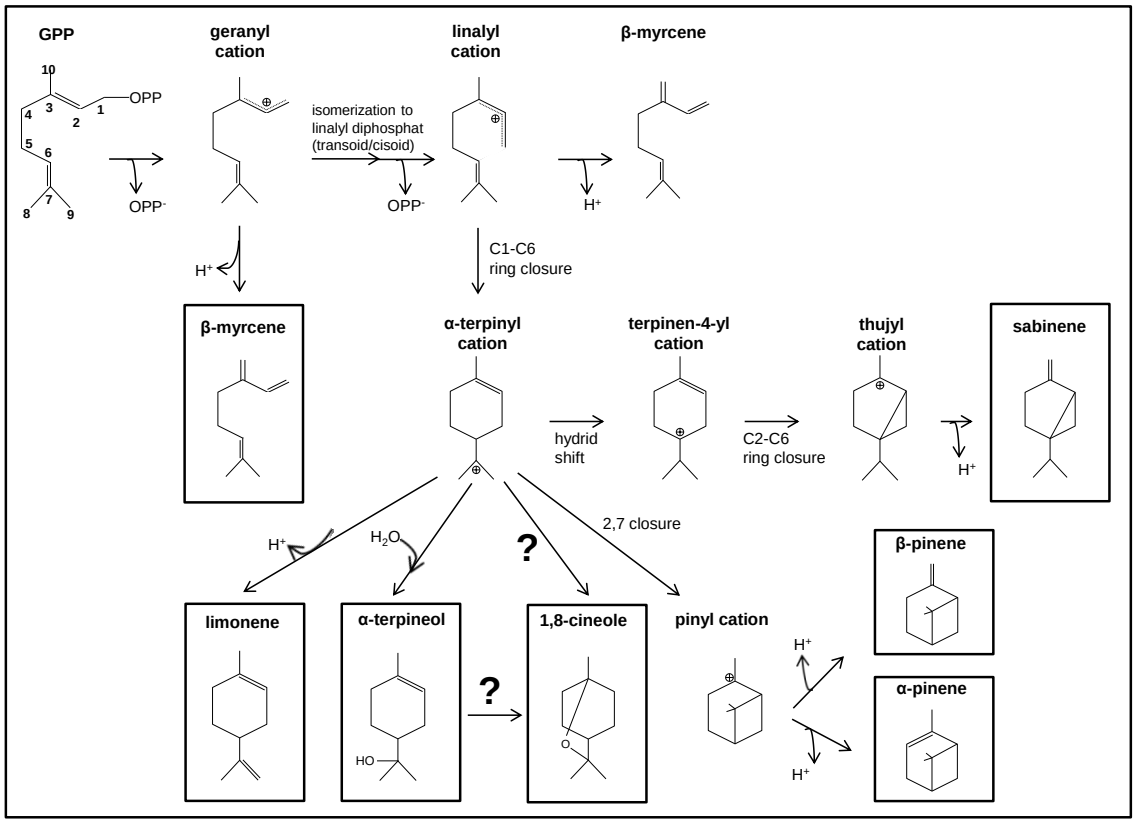


Figure 5: Synthesis of "cineole cassette" monoterpenes. The substrate geranyl pyrophosphate (GPP) is ionized by diphosphate elimination to give the geranyl cation. Subsequently, this cation is converted into the linalyl cation and α-terpinyl cation. The carbocation intermediate α-terpinyl cation is the precursor for all cyclic monoterpenes and via hydratisation or deprotonation α-terpineol and limonene are formed. The 2,7 closure results in the pinyl cation which is deprotonated to give β-pinene and α-pinene. Sabinene is synthesized via two additional carbocations. The synthesis to β-myrcene can be realized via the geranyl cation or linalyl cation by deprotonation. The mechanism to 1,8-cineole is questionable. It could be either formed via α-terpineol or from the α-terpinyl cation. (Degenhardt et al. 2009, modified).

1.2 Monoterpene synthases are special enzymes

In general, terpene synthases are divided into six terpene synthase subfamilies (*Tps*a – f) based on phylogenetic analyses of the amino acid sequence (Bohlmann et al. 1997). Angiosperm monoterpene synthases belong to the subfamily *Tpsb,* and gymnosperm monoterpene synthases belong to *Tpsd*. Monoterpene synthases have a length of 600 to 650 amino acids and a molecular weight of 50-80 kDa (Bohlmann et al. 1998). Monoterpene synthases are class I enzymes, which are ionization-dependent terpenoid cyclases (Wendt & Schulz 1998; Christianson 2006). The class I enzymes contain a DDXXD motif and bind the pyrophosphate group of the substrate via magnesium ions to facilitate the release of the pyrophosphate group (Wendt & Schulz 1998).

Monoterpene synthases often consist of α-helices, and this chain fold has been designated the class I terpenoid synthase fold. However, prenyl transferases and sesquiterpene synthases also exhibit the class I terpenoid synthase fold. Class II terpenoid synthases, e.g., tri- and tetraterpene cyclases, activate the substrate by protonation to generate the carbocation intermediates (Wendt & Schulz 1998). All monoterpene synthases require divalent metal ions as essential co-factors. The type of divalent metal ions used by the enzyme depends on whether the plant is an angiosperm (magnesium ion) or gymnosperm (manganese ion) (Bohlmann et al. 1998). Prenyltransferases from the beetle larvae *Phaedon cochleariae* also employ divalent cobalt ions as co-factors (Frick et al. 2013).

Monoterpene synthases are special terpene enzymes than can synthesize a multitude of monoterpenes and monoterpenoids from a single substrate (Tholl 2006; Degenhardt et al. 2009). Enzymes that are similar in sequence synthesize cyclic or acyclic compounds, have different product spectra and are single- or multi-product enzymes (Maruyama et al. 2001). The isolated α-terpineol synthase from grape vine converts GPP into 14 monoterpenoid compounds (Martin & Bohlmann 2004), while the enzymes from *Pinus* spp. and from *Zea mays* convert GPP into six monoterpenoids (Phillips et al. 2003; Lin et al. 2008). Single-product monoterpene synthases have also been isolated, e.g., α-terpineolsynthase from *Magnolia grandiflora* (Lee & Chappell 2008), (*E*)-β-ocimene synthase from *Medicago truncatula* (Navia-Giné et al. 2009) and the β-myrcene synthases from *Humulus lupus* and *Oryza sativa* (Wang et al. 2008, Yuan et al. 2008). Compared to sesquiterpene synthases (comprise C_{15} bodies), monoterpene synthases synthesize a low number of multiple compounds. A sesquiterpene synthase from *Abies grandis* (γ-humulene synthase) catalyzes the formation of 52 sesquiterpenes from the substrate farnesyl pyrophosphate (FPP) (Steele et al. 1998). Nevertheless, the investigated monoterpene synthase in this study are the 1,8-cineole synthase from *N. suaveolens* (Roeder et al. 2007) and the α-terpineol synthase from *N. langsdorfii* (Fähnrich et al. 2011). Both enzymes synthesize five “cineole cassette” monoterpenes (sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol) but with different major compounds. 1,8-cineole is the major

compound of the 1,8-cineole synthase of *N. suaveolens* and α-terpineol is the major compound of the α-terpineol synthase from *N. langsdorfii*.

Multi-product formation is not fully understood, and the amino acids in the active pocket of monoterpene synthases have not been identified. However, it is known that special motifs in the sequence of monoterpene synthases have different functions. The protein sequence of monoterpene synthases starts with a special N-terminal transit peptide that is essential for transporting the core encoded monoterpenoids in the plastids (Schmidt et al. 1979; Turner et al. 1999). The preprotein monoterpene synthases are converted to the mature form after transport (Keegstra et al. 1989; Williams et al. 1998). The transit peptide is proteolytically degraded by a chloroplast processing enzyme (Richter & Lamppa 1998). The N-terminal localized $RR(X)_8W$ motif plays a role in the isomerization of the substrate GPP (Williams et al. 1998). A downstream localized RWW motif and C-terminal localized CYMNE motif have been identified in several monoterpene synthases of *Nicotiana* species (*N. suaveolens, N. forgetiana*, *N. langsdorfii, N. alata*), but the functions of these motifs are not yet known (Roeder et al. 2012; Fähnrich et al. 2011, 2012). The RDR motif is important for protecting the carbocation intermediate against nucleophilic attack after ionization of the substrate (Starks et al. 1997). Crystallization and mutational analyses of monoterpene synthases from *Salvia* species have demonstrated that the NALV motif is responsible for the product outcome and enzyme specificity (Kampranis et al. 2007). The aspartate-rich DDXXD motif and short (N,D)D(L,I,V)X(S,T)XXXE “NSE/DTE” motif play important roles in the coordination and binding of the divalent metal ions to the trinuclear magnesium cluster. This cluster supports substrate ionization and following the cyclization reaction to the α-terpinyl cation (Tarshis et al. 1994; McGarvey & Croteau 1995; Starks et al. 1997; Bohlmann et al. 1998; Christianson et al. 2006; Cane & Kang 2000).

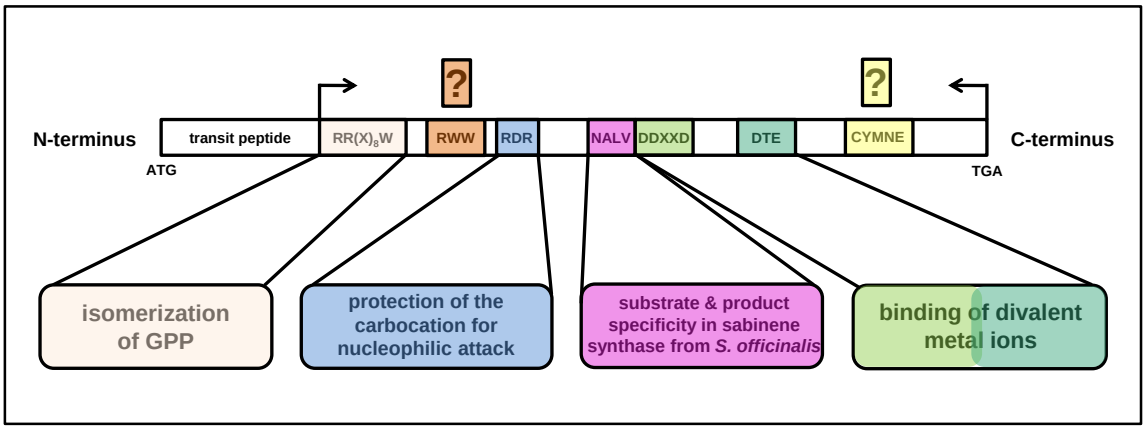


Figure 6: Conserved motifs in the sequence of monoterpene synthases. At the N-terminus is localized in light pink the $RR(X)_8W$ motif: important for the isomerization of the substrate GPP, brown: functionally unknown RWW motif, blue: RDR motif which protects the carbocation from nucleophilic attack. At the C-terminal end is localized in pink the NALV motif, which is important for the substrate and product specificity as described for the sabinene synthase (Kampranis et al. 2007), green: DDXXD motif and cyan blue: DTE motif both essential to bind the divalent metal ions, yellow: functionally unknown CYMNE motif. ATG and TGA mark the start and stop codon.

1.3 The genus *Nicotiana*

Several species of the genus *Nicotiana* synthesize and emit a broad range of terpenes, particularly monoterpenes. Raguso et al. (2003) analyzed the volatile compounds emitted by different *Nicotiana* species, e.g., *N. suaveolens, N. sylvestris, N. alata,* and *N. longiflora*. All these species emit a broad range of monoterpenes, including aromatic esters (e.g., methyl benzoate, methyl salicylate) and nitrogenous compounds (e.g., methyl nicotinate). The synthesis of multitude monoterpenes indicates the presence of monoterpene synthases in different *Nicotiana* species, as confirmed by the isolation of multiple monoterpene synthases, sesquiterpene synthases and diterpene synthases from *Nicotiana* species.

The genus *Nicotiana* was named for the French diplomat Jean Nicot because he is believed to have brought tobacco seeds from Portugal to France (Knapp et al. 2004). *Nicotiana* belongs to the family *Solanaceae* and is a member of the world's most important agricultural species, in addition to tomatoes, potatoes, chili peppers (Olmstead et al. 2008). The genus *Nicotiana* is divided into three subgenera, *Tabacum, Rustica* and *Petunioides*, based on cytological and morphological characteristics (Goodspeed 1954). The 76 naturally occurring species are divided into 13 sections: *Alatae, Nicotiana, Noctiflorae, Paniculatae,*

Petunoides, Polydicliae, Repandae, Rusticae, Suaveolentes, Sylvestres, Tomentosae, Trigonophyllae and *Undulatae* (Knapp et al. 2004). Species of sections *Suaveolentes* and *Alatae* were examined in the present study. Most *Nicotiana* species (e.g., *N. langsdorfii* or *N. forgetiana*, both members of the section *Alatae*) originated in South America (e.g., Argentina, Brazil, Chile; Figure 7) (Goodspeed 1954). *Nicotiana* species were distributed to other regions by continental drift, as described by Alfred Wegener (Wegener, 1912), and by humans. The species *N. suaveolens*, which is eponymous for the section *Suaveolentes*, is widespread in Australia and includes an endemic species, *N. africana*, that is distributed in Nambia (Merxmüller & Buttler, 1975).

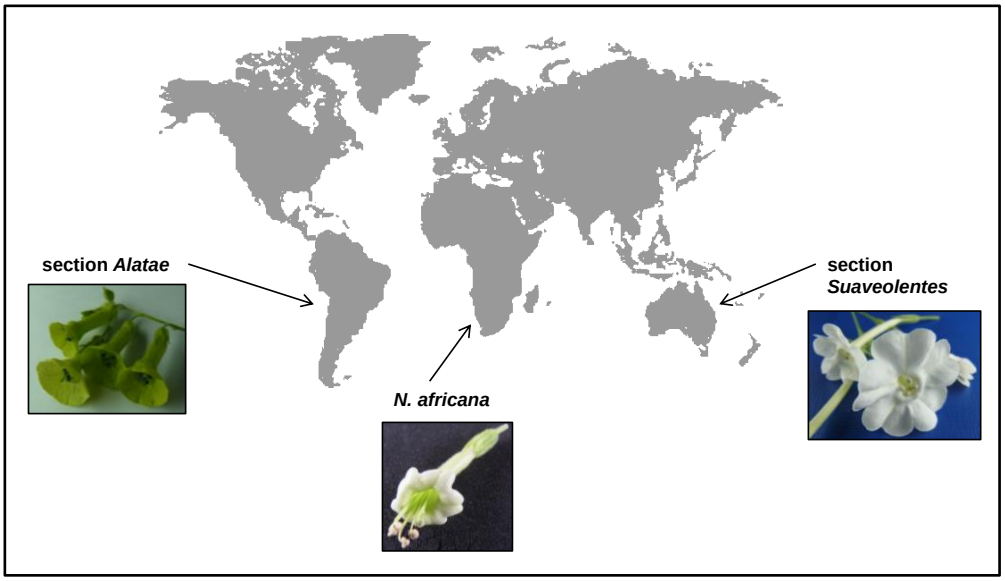


Figure 7: Distribution of *Nicotiana* section *Alatae* and *Suaveolentes*. Species of the section *Alatae* are distributed in South America. The picture shows flowers of *N. langsdorfii*. The section *Suaveolentes* (picture: flowers of *N. suaveolens*) is native to Australia and contains an endemic specie, *N. africana*, found only in Namibia.

The morphology of *Nicotiana* is very variable and includes small flowers with short tubes (e.g., *N. obtussifolia*) and large flowers with long tubes (e.g., *N. suaveolens, N. longiflora*). The stylus is located in the inner part of the flower (*N. alata*) or outside the flower (*N. africana*). The petal color of *Nicotiana* species includes white, green, yellow, pink and red. The plants grow in a herbaceous manner and, in the case of *N. mutabilis*, can be very tall (up to 5 ft). The type of pollination differs among species. *N. longiflora* are self-pollinated, whereas *N. suaveolens* and *N. alata* are pollinated by the hawkmoth (Raguso et al. 2003; Ippolito et al. 2004). *N. langsdorfii* is pollinated by hummingbirds

(Ippolito et al. 2004). The type of pollination is determined not by flower morphology but by volatile compounds emitted, such as terpenes and terpenoids that are the focus of this study. Pollination by insects (e.g., hawkmoth) depends on the volatile scent bouquet (Loughrin et al. 1990; Raguso et al. 2003). Species of the section *Alatae* primarily emit monoterpenes representing the “cineole-cassette” (Raguso et al. 2003). Species of section *Suaveolentes* emit more nitrogenous compounds, aromatic alcohols, aldehydes and small amounts of “cineole-cassette” monoterpenoids, with the exception of *N. suaveolens*, which primarily emits compounds of the “cineole-cassette” (Roeder et al. 2007). Roeder et al. (2007) isolated a 1,8-cineole synthase from *N. suaveolens*. Fähnrich et al. (2011, 2012) isolated a 1,8-cineole synthase from *N. forgetiana* and a α-terpineol synthase from *N. langsdorfii.* The species *N. langsdorfii* belongs to the same section as *N. forgetiana* (section *Alatae*). Phylogenetic analysis demonstrated that the 1,8-cineole synthases of section *Alatae* (*N. forgetiana*) and *Suaveolentes* (*N. suaveolens*) cluster together, contradicting the assumption that taxonomically related species cluster together (Fähnrich et al. 2012). Figure 8 presents an alignment at the amino acid level of the 1,8-cineole synthases from *N. suaveolens* and *N. forgetiana* and the α-terpineol synthase from *N. langsdorfii.* Ten amino acid differences were detected between the 1,8-cineole synthases from *N. forgetiana* and the α-terpineol synthase from *N. langsdorfii*, whereas the 1,8-cineole synthases from *N. forgetiana* and *N. suaveolens* differed in 50 amino acids. The comparison of the 1,8-cineole synthase from *N. suaveolens* and the α-terpineol synthase from *N. langsdorfii* revealed 53 amino acid differences. This analysis demonstrated that catalytically similar enzymes cluster together (Fähnrich et al. 2012).

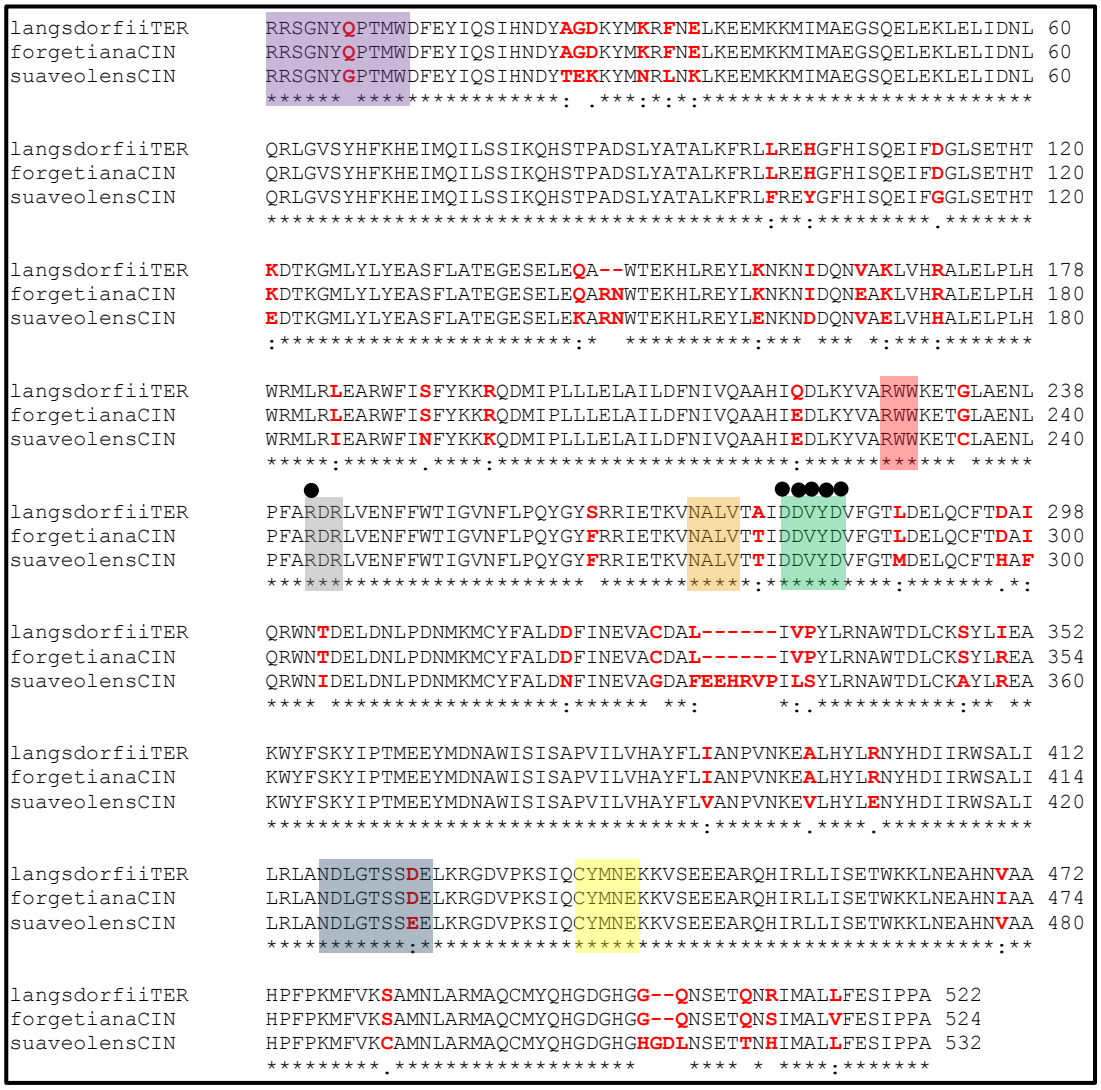


Figure 8: Amino acid sequence alignment of the 1,8-cineole synthases of *N. suaveolens* and *N. forgetiana* and the α-terpineol synthase of *N. langsdorfii*. All differences between the enzymes are highlighted in red. The yellow background marks the similar amino acids between α-terpineol synthase of *N. langsdorfii* and 1,8-cineoles synthase of *N. forgetiana* and the difference of both enzymes to 1,8-cineole synthase of *N. suaveolens*. Asterisks (*): same amino acid, spaces: amino acids completely different, double dots: amino acids differ from each other but have similar properties, one dot: somewhat similar properties (EMBL-EBI 2014). The sequence alignment was done with CLUSTALW 2.0.10 (Thompson et al., 1994).

1.4 Objective

The 1,8-cineole synthase from *N. suaveolens* synthesizes 1,8-cineole as the major monoterpenoid and sabinene, limonene, β-myrcene and α-terpineol as by-products. However, the α-terpineol synthase from *N. langsdorfii* produces α-terpineol as the major compound and sabinene, limonene, β-myrcene and 1,8-cineole as by-products. These enzymes are multi-product monoterpene synthases that both produce five monoterpenes but with different product distributions. One aim of this study was to investigate the mechanism underlying the synthesis of either more 1,8-cineole or α-terpineol. Accordingly, the 1,8-cineole synthase from *Nicotiana suaveolens* (Roeder et al. 2007) and the α-terpineol synthase from *N. langsdorfii* (Fähnrich et al. 2011) were aligned, and amino acids that differs between the sequences should be mutated by site-directed mutagenesis. One or multiple amino acids may be responsible for the synthesis of more 1,8-cineole than α-terpineol. This study analyzed the mechanism of the cineole synthase reaction to determine how the enzyme synthesizes 1,8-cineole as well as multiple products. Furthermore, the amino acids in the catalytically active pocket should be analyzed. The active pocket is putatively located near the DDXXD motif, but conserved amino acids have not been identified. The molecular studies and generation of mutant enzymes should be complemented by the determination of the crystal structure of the 1,8-cineole synthase of *N. suaveolens*.

2. Material and methods

2.1 The experimental work flow from site-directed mutagenesis to enzyme activity measurements

The experimental steps from mutagenesis till enzyme analysis are shown in Figure 9. The generation of 19 mutants started with site-directed mutagenesis (Figure 9, A). The PCR required mutated primer pairs and double stranded plasmid (supplement 1). DNA gel electrophoresis was conducted in order to check the double stranded, mutated plasmid regarding amplification and accurate size. All generated mutants had the same size of 7,2 kbp comprising the Champion^{TM} pET SUMO vector (5,6 kbp) and the fragment of the 1,8-cineole synthase (1,6 kbp) (Figure 9, B). The plasmid was then transformed into *E. coli*. Plasmid DNA of positive transformants was sequenced to validate if the mutation is at the correct and desired position in the gene sequence and no other mutation occurred compared to the wild type sequence. The complete nucleotide and amino acid sequences of every mutant and their alignment with the wild type are documented in supplement 2 and 3. A positive clone of each mutation was heterologous overexpressed in *E. coli* HMS174 (DE3) (2.1.2). The crude extract (10 µg total protein) was used to perform an enzyme assays to check their activities and to determine their product profiles (Figure 9, C). The α-terpineol/1,8-cineole ratio was calculated for each mutated enzyme and compared with the wild type. The α-terpineol / 1,8-cineole ratio describes the efficiency of the cyclization reaction (Fähnrich et al. 2012). If the enzyme in the crude extract showed activity and also an interesting result concerning an altered α-terpineol / 1,8-cineole ratio it was purified by Ni^{2+}-NTA agarose affinity chromatography. The elution steps were performed and the fraction with the highest protein concentration was used for an enzyme assay with non-radioactive or radioactive GPP to determine the catalytical efficiency and activity. The purified enzyme was always more concentrated than the enzyme in the crude extract. In some cases the purified enzyme lost the activity despite the fact that it was active in the crude extract. For statistical reasons and to be able to calculate significances, the crude extract was analyzed two or more times. The SDS-PAGE and Western Blot with specific antibodies (Figure 9, D)

provided evidence that the enzyme existed in respective fraction. Every mutated enzyme had the same molecular weight of ~ 68 kDa.

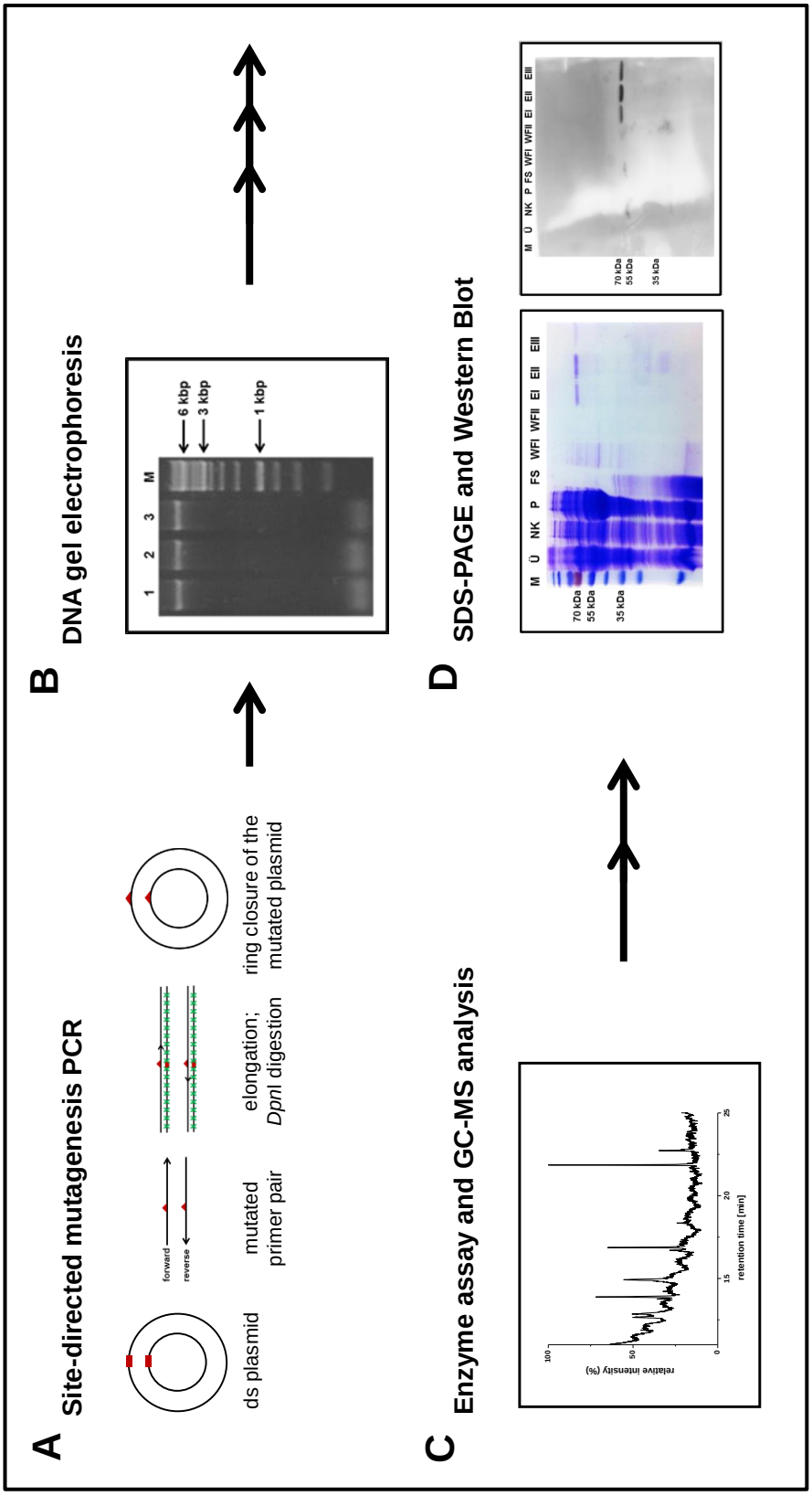


Figure 9: Illustration of the working procedure. **A**: Site-directed mutagenesis PCR was used to generate the mutants of the 1,8-cineole synthase from *N. suaveolens*. **B**: PCR products of mutant plasmid DNA were analyzed by DNA gel electrophoresis. The next steps were: transformation of plasmid DNA into *E. coli*, gene was sequenced to verify correct sequence, heterologous overexpression in *E. coli*. **C**: Enzyme assay and GC-MS analysis of the *E. coli* headspace volatiles. **D**: SDS-PAGE and Western Blot were used to analyze the overexpressed protein. Specific antibodies identified the overexpressed and affinity chromatography purified enzyme (more details: material and methods).

2.1.1 Site-directed mutagenesis to generate mutant enzymes of the 1,8-cineole synthase

The plasmid Champion™ pET sumo protein vector (Invitrogen) contains the gene, which encodes for the 1,8-cineole synthase. This was used as the template for mutagenesis PCR with special mutated primer pairs (supplement 1). The PCR was performed using a modified protocol (supplement 1) of the „QuikChange II XL Site-Directed Mutagenesis Kit“ (Agilent Technologies). After digestion with *Dpn*I (Thermo Fisher Scientific) and the transformation into *E. coli* HMS174 (DE3) the mutants were verified by sequencing the complete gene construct (GATC Biotech AG). The biological replicates are marked with n_{biol} and technical replicates marked with n.

2.1.2 Heterologous overexpression and purification

Clones of *E. coli* HMS174(DE3) transformed with the vector Champion™ pET sumo protein vector (Invitrogen)carrying the wild type gene or the mutated genes of the 1,8-cineole synthase from *N. suaveolens* were grown overnight by 37 °C and 170 rpm (IRC-1-U Clim-O-Shake® System Kühner, Adolf Kühner AG Apparatebau) in a preculture with LB medium containing 50 µg/mL kanamycin. The main culture was prepared in a LB-medium flask relation 1:5 and inoculated with 1 mL of the preculture and supplemented with 1 M glucose (final concentration: 3 mM) and the appropriate antibiotic (50 µg/mL kanamycin). The culture was grown at 37 °C, 170 rpm and induced with 1 M Isopropyl-β-$_D$-thiogalactopyranoside (IPTG, final concentration: 0.5 mM) after reaching an OD_{600} of 1. The overexpression conditions were 20 °C by 175 rpm for 20 h. After induction the cells were harvested by centrifugation for 15 min at 4 °C at 8,000 g (Sigma 3K30, Sigma) and the pellet was resuspended in 2 mL/g lysis buffer (Iijima et al. 2004) (50 mM TRIS (pH 7.5), 10 % glycerol (w/v), 10 mM β-mercaptoethanol). The cell lysis was performed by incubating the cell suspension with 50 mg/mL lysozyme (final concentration: 1 mg/mL) for 2 h on ice and after that sonication for 5 x 20 sec. The following centrifugation step for 30 min at 11,000 g and 4 °C separated the cell debris from protein-containing

the soluble fraction. The soluble fraction was passed through a column filled with Ni^{2+}-NTA agarose (QIAGEN, Germany) and purified by affinity chromatography. The matrix was equilibrated with 10 mL Aqua dest. and 10 mL buffer A (50 mM TRIS, 500 mM NaCl, 20 mM imidazole, 10 % glycerol, pH 7.5 and fresh supplemented with 1 % Tween and 0.1 % β-mercaptoethanol). Subsequently, the soluble fraction was incubated for 1 h on the column matrix on ice and on a rotary shaker. After incubation the purification steps started with washing the column first with 5 mL and following with 10 mL of buffer A. The protein was eluted with three times 500 µl of buffer B (50 mM TRIS, 500 mM NaCl, 250 mM imidazole, 10 % glycerol, pH 7.5 and fresh supplemented with 0.1 % β-mercaptoethanol). The protein concentration was measured using Bradford (Bradford 1976) and an enzyme assay was prepared to validate the activity and the enzymatic kinetic. In addition, the overexpression of the protein was verified using SDS-PAGE and Western Blot.

2.1.3 SDS-PAGE and Western Blot

After purification of the proteins by Ni^{2+}-NTA agarose 18 µl of every protein fraction were applied on a 12.5 % polyacrylamide gel. The samples were mixed with 3 µl 5 x SDS loading buffer (100 mM TRIS, 20 % glycerin, 4 % SDS, 200 mM DTT, 5 % β-mercaptoethanol, 0.2 % bromophenol blue) and the proteins were electrophoretically separated at 135 V for 2 h (Miniprotean, Bio-Rad Laboratories, USA). The gels were stained with Coomassie Brilliant Blue R-250 to visualize the proteins or transferred on a PVDF (polyvinyldifluoride) membrane to perform a Western Blot. The membrane was firstly incubated with a primary antibody (1:3,000 dilution in blocking solution (4 % skim milk pouder, 1 % albumin fraction V, *ad*. 1 x TBS-Triton X-100) against the polyhistidin followed by a secondary antibody was conjugated with the alkaline phosphatase (1:20,000 dilution in 1 x TBS-Triton X-100, Sigma-Aldrich, St. Louis, USA). To detect the proteins, CDP-Star (1:200 dilution in detection buffer (100 mM TRIS, 150 mM NaCl, 50 mM $MgCl_2$) was used as substrate for the alkaline

phosphatase. The luminescence was analyzed and quantified with using AIDA Image Analyzer Software (Raytest Stella Isotopenmeßgeräte, Deutschland).

2.1.4 Nonradioactive enzyme assay and GC-MS analysis

The enzyme assay was prepared with the 10 µg crude extract or 10 µg of the purified enzyme. GPP (1 mg/mL, Echelon Biosciences, Salt Lake City) was used as substrate and the assay was performed with 5 x Hepes buffer (250 mM Hepes, 100 mM $MgCl_2$, 2.5 mM $MnCl_2$, 50 % glycerol, pH 8.0) and 0.1 M dithiothreitol (DTT, final concentration: 2 mM) in 0.2 mL total volume. The aqueous phase was overlaid with 200 µL hexane containing 5 ng/µL cis-nerolidol as internal standard. The assay was incubated for 3 h at 41.5 °C. The synthesized products were transferred into the hexane phase (shaking and separating) and analyzed using gas chromatography coupled with a mass spectrometer (GC-MS, Shimadzu QP5000). One µL of the sample was splitless injected and separated on a DB5-MS column (60 m x 0.25 mm x 0.25 mm; J+W Scientific Folsom, California, USA). Helium was used as carrier gas. The column flow was set to 1.1 mL/min at a column inlet pressure of 123.1 kPa. The temperature program started with 35 °C hold for 2 min and the temperature gradient increased in steps of 10 °C per minute up to 280 °C (holding time: 15 min). The total program time for one sample run was 41.50 min. Mass spectra were acquired in the range of *m/z* 40 – 280. For compound identification retention indices and mass spectra were compared with those that are available in the library of the National Institute of Standards and Technology (NIST Standard Reference Database 147) and reference compounds.

2.2 Determination of the enzymatic parameters K_M, V_{max} und k_{cat}

For the biochemical characterization of enzymes, the catalytically efficiency is essential to determine the substrate specificity and the reaction rate. Different substrate concentrations (10 nM, 20 nM, 30 nM, 40 nM, 50 nM, 60 nM) were chosen to determine the product formation rate for every substrate

concentration for different time points (2 min to 10 min). The initial rate (linear range) of each substrate concentration was plotted in a diagram. To define the parameters K_M und V_{max} the Lineweaver-Burk kinetic was used. The assay was performed in a total volume of 50 µl with 10 µl 5xHepes buffer, 1 µl substrate ^{3}H-GPP (1 mCi/ml, Hartmann Analytics, Braunschweig), 1 µg enzyme, 2.5 mM DTT and overlaid with 180 µl hexane and incubated at 41.5 °C. The reaction was stopped by transferring it on ice (15 sec), mixing (1 min) and centrifugation for 2 min at 13000 rpm. 50 µl of the organic phase was pipetted to 2 ml scintillation solution (Ultima Gold^{TM}, Perkin Elmer) and the product formation was read using a Liquid scintillation analyzer (Tri-carb 2810 TR, Perkin Elmer).

2.3 3D modelling

The predicted protein model of the 1,8-cineole synthase from *N. suaveolens* (Roeder et al. 2007) was created on the basis of homology modelling with a crystallized enzyme as template. The limonene synthase from *Mentha spicata* (Hyatt et al. 2007) was crystallized and had 46 % sequence similarity to the 1,8-cineole synthase. At least 30 % sequence similarity is strongly recommended to perform the 3D protein modelling. The program Swiss PdB viewer 4.01 (Guex & Peitsch 1997) was chosen to model a hypothetical 3D crystal structure. Therefore the protein sequences of both enzymes were aligned using CLUSTALW 2.0.10 (Thompson et al. 1994) and gaps were removed. The generated structure of the 1,8-cineole synthase was saved as a pdb file and upload in the program PyMOL (The PyMOL Molecular Graphics System, Version 0.98rc5 Schrödinger, LLC).

3. Results

The aim of this study was to determine the reaction mechanism by which the 1,8-cineole synthase from *N. suaveolens* converts α-terpineol to 1,8-cineole. This multi-product monoterpene synthase synthesizes four monoterpenoids (sabinene, β-myrcene, limonene, α-terpineol) in addition to 1,8-cineole as the major compound. The α-terpineol synthase from *N. langsdorfii* also catalyzes the formation of five monoterpenoids, but α-terpineol is the major compound. To determine if the amino acid sequences of these enzymes are responsible for the differences in product formation, both amino acid sequences were aligned, and selected amino acids in the 1,8-cineole synthase from *N. suaveolens* were substituted by site-directed mutagenesis.

3.1 Selection of amino acids in the 1,8-cineole synthase from *N. suaveolens* that influence the conversion of α-terpineol to 1,8-cineole

The comparison of the 1,8-cineole synthase from *N. suaveolens* (Roeder et al. 2007) with the α-terpineol synthase from *N. langsdorfii* (Fähnrich et al. 2011) revealed 53 differences at the amino acid level (Figure 10 A). Site-directed mutagenesis was used to identify essential amino acids involved in the putative reaction mechanism to 1,8-cineole.

Particular focus was given to amino acids localized at the C-terminus of the protein and near the DDXXD motif in the amino acid sequence as well as in the nearest layer of amino acids visualized in the protein model (Figure 10 B). The DDXXD motif located at the C-terminus of the enzyme (Figure 10 B) is essential to coordinate and bind the divalent metal ions (e.g., Mg^{2+}, Mn^{2+}, Co^{2+}). Metal ions are necessary co-factors of the enzyme and are important for activity (Bohlmann et al. 1998; Christianson 2006, Starks et al. 1997). Therefore, the DDXXD motif may play a relevant role in the putative active pocket, and amino acids near and around this motif may affect the cyclization reaction to 1,8-cineole.

The mutations M290L, H298D, M290L/H298D, F300I and A355S are in close proximity to the DDXXD motif (Figure 10 A and B and Table 1) and were generated to determine the influence of these residues on the cyclization reaction to 1,8-cineole. These mutations may affect the steric structure of the enzyme to disrupt metal ion coordination, thereby affecting the reaction to 1,8-cineole or resulting in its termination in the production of α-terpineol.

The mutation T279A (Table 1) was generated because of its location between the NALV motif and the DDXXD motif. The NALV motif (Figure 10) was identified as important for product specificity in the sabinene synthase from *S. officinalis* (Kampranis et al. 2007), and mutations near this motif may also influence the product outcome of the terpene synthase from *Nicotiana suaveolens.*

The substitutions I305T and N324D are distant from the DDXXD motif (see the 3D protein model in Figure 10 B) compared to the other mutants described above, but several studies have demonstrated that amino acids in the surrounding layers of an active pocket can influence enzyme activity (Back & Chappell 1996; Starks et al. 1997; Hyatt & Croteau 2005) and, possibly, the product outcome.

A double mutant in which the glutamate residues at positions 335 and 336 were deleted was generated to assess the significance of these two amino acids, which are present in the sequence of the 1,8-cineole synthase from *N. suaveolens* but not the α-terpineol synthase gene of *N. langsdorfii* (Figure 10 B and Table 1). These additional amino acids may be crucial for the synthesis of more 1,8-cineole, consistent with the hypothesis that a hydroxyl group in the side chain of an amino acid protonates the double bond of α-terpineol via a carbocation intermediate to form 1,8-cineole (Bartelt 2014). The mutations Y446A and Y502V (Figure 10 B and Table 1) were generated to determine the influence of these residues on the possible protonation of the double bond of the α-terpineol to initiate the formation of 1,8-cineole.

The substitutions G7Q, G7Q/M290L and C235G are located N-terminal to the DDXXD motif (Figure 10 B). These mutants were generated to determine if the N-terminal part of the enzyme influences the reaction to 1,8-cineole. The substitution G7Q is localized in the $RR(X)_8W$ motif, which participates in the isomerization of the substrate GPP to form a cyclic intermediate (Williams et al.

1998; Degenhardt et al. 2009). It was assumed that replacement with a glutamine will influence the formation of the bicyclic product 1,8-cineole or cyclic products in general. The double mutant G7Q/M290L was generated because these substitutions are near two sequence motifs that are essential for product synthesis and enzyme activity. The mutation C235G is near the functionally uncharacterized RWW motif and the RDR motif. Peters & Croteau (2002) proposed that the first arginine of the RDR motif might function in binding and stabilizing the released diphosphate of the substrate GPP. Mutational analysis of the RXR motif in γ-humulene synthase from *Abies grandis* disturbed diphosphate binding and resulted in the formation of many products, similar to mutations in the DRRYR motif of the trichodiene synthase from *Fusarium sporotrichioides* (Cane et al. 1995). Therefore, it was hypothesized that a mutation close to this motif might influence the cyclization to 1,8-cineole due to steric alterations.

The mutation F266S, which is also N-terminal to the DDXXD motif (Figure 10), was generated because the results obtained for the mutant S264F of the α-terpineol synthase from *N. langsdorfii* (Klodt, 2013). Klodt (2013) demonstrated that this mutation altered the product profile of the α-terpineol synthase from *N. langsdorfii* toward synthesis of more 1,8-cineole. To confirm this effect, the reverse mutation F266S in the 1,8-cineole synthase from *N. suaveolens* was constructed. Four other mutations (F266T, F266V, F266Y and F266C) were also generated to analyze the reaction mechanism of 1,8-cineole formation. Table 1 provides an overview of all generated mutations, their location in the gene sequence and the changes in amino acid properties.

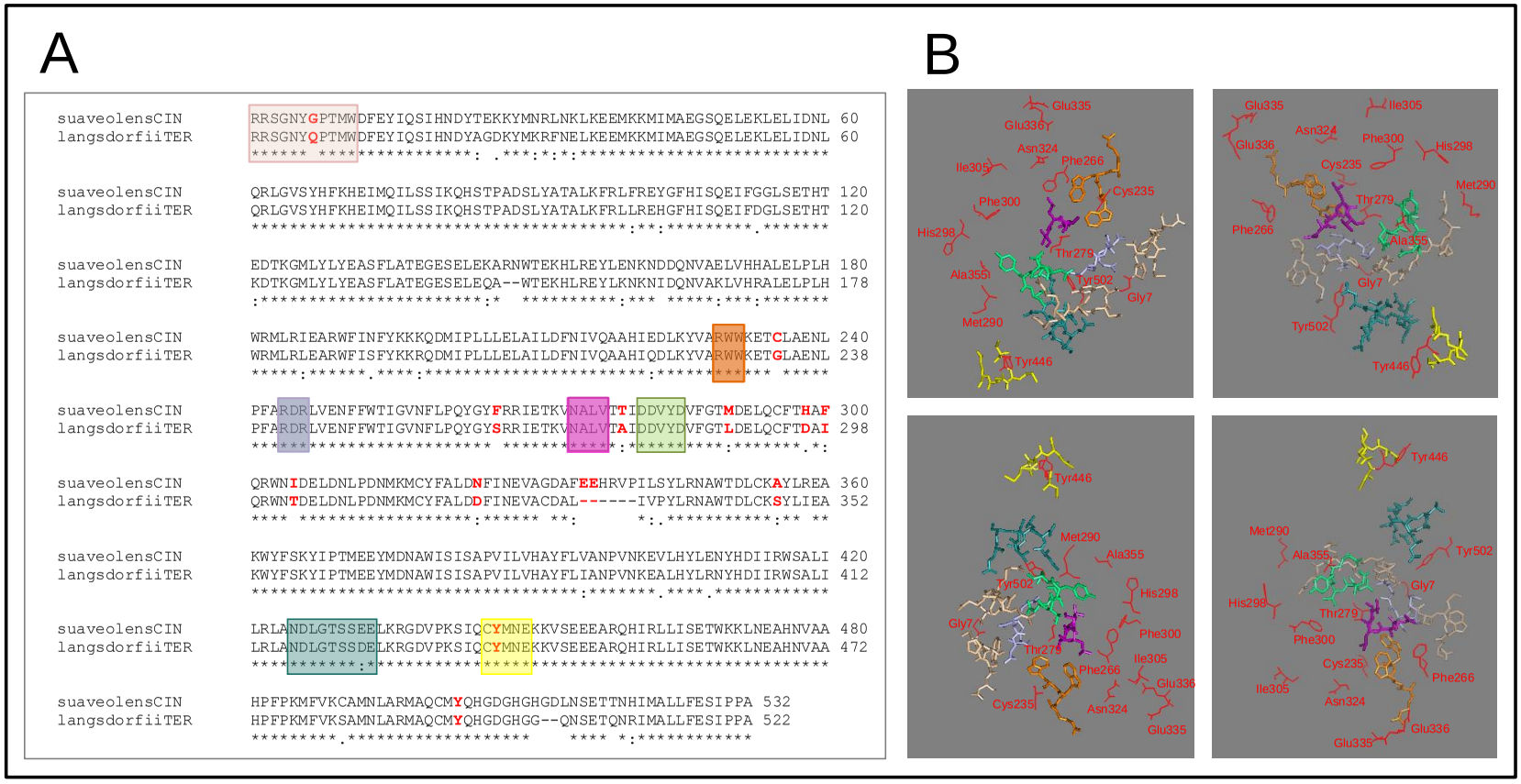


Figure 10: Sequence of the 1,8-cineole synthase from *N. suaveolens* aligned with the α-terpineol synthase from *N. langsdorfii*. A: Conserved sequence motifs are coloured in green: DDxxD motif, yellow: CYMNE motif, cyan blue: NSE/DTE motif, light blue: RDR motif, pink: NALV motif, orange: RWW motif, wheat: RR(x)$_8$W motif. All generated mutants are highlighted in red. Asterisks (*): same amino acid, spaces: amino acids different, double dots: amino acids differ from each other but with similar properties, one dot: weakly similar properties (EMBL-EBI 2014). The sequence alignment was done with CLUSTALW 2.0.10 (Thompson et al., 1994). B: Four perspectives of the stick model of the 3D structure of the 1,8-cineole synthase from *N. suaveolens*. The coloured molecules are the conserved sequence motifs and refer to the colour code in A. The residues are shown in red. The numbers of the amino acids refer to the position in the sequence of the 1,8-cineole synthase. The 3D structure of the protein model was performed by using Swiss PdB Viewer 4.01 (Guex and Peitsch 1997) and PyMOL v0.98rc5 (Schrödinger LLC).

Table 1: Mutants of the 1,8-cineole synthase from *N. suaveolens*. The abbreviation, the localization of the mutation in the protein and the change of the property of the amino acids are given.

mutant	location of the mutation	change in amino acid properties
G7Q	N-terminal end	nonpolar to polar
G7Q/M290L		nonpolar to polar/remains nonpolar
C235G	middle part of the enzyme	polar to nonpolar
F266S		nonpolar to polar
F266C		nonpolar to polar
F266Y		nonpolar to polar
F266T		remains nonpolar
F266V		nonpolar to polar
T279A		polar to nonpolar
M290L		remains nonpolar
H298D		polar to negative charged
M290L/H298D		remains nonpolar/ polar to negative charged
F300I		remains nonpolar
I305T		nonpolar to polar
N324D		polar to negative charge
EE335/336-deleted		negative charge
A355S		nonpolar to polar
Y446A	C-terminal end	polar to nonpolar
Y502V		polar to nonpolar

3.2 Analysis of the wild type 1,8-cineole synthase from *N. suaveolens*

The wild type 1,8-cineole synthase was biochemically analyzed, and the results are summarized in Figure 11. The wild type enzyme in the crude extract and in the purified fraction synthesized five monoterpenoid compounds: sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol. The major compound of this multi-product enzyme was 1,8-cineole. The average specific activity of the 1,8-cineole synthase was 62 pkat/mg in the crude extract and 3093 pkat/mg in the purified faction (Figure 11). Based on the other products synthesized, the following specific activities were calculated: α-terpineol (26.4 pkat/mg in the crude extract and 1149.9 pkat/mg in the purified fraction), β-myrcene (11.5 pkat/mg in the crude extract and 434.7 pkat/mg in the purified fraction), limonene (7.5 pkat/mg in the crude extract and 381.1 pkat/mg in the purified fraction) and sabinene (6.6 pkat/mg in the crude extract and 374.1 pkat/mg in the purified fraction). The analyzed crude extract (114 pkat/mg) had a lower total specific activity than the purified enzyme (5433.2 pkat/mg). The specific activities of the purified 1,8-cineole synthase from *N. suaveolens* were significantly higher than the specific activities of the recombinant 1,8-cineole synthases from *N. bonariensis*, *N. forgetiana* and *N. longiflora* (Fähnrich et al. 2012) because 1,8-cineole synthase activity was 120 – 270 pkat/mg, α-terpineol synthase activity was 45 – 125 pkat/mg, and the activity corresponding to by-product synthesis (sabinene, limonene, β-myrcene, α- and β-pinene) was less than 60 pkat/mg (Fähnrich et al. 2012).

The percentage distributions of the compounds synthesized by the 1,8-cineole synthase from *N. suaveolens* in the crude extract and as the purified enzyme were nearly identical (Figure 11). The α-terpineol/1,8-cineole ratio was slightly higher for the purified enzyme (1:2.7) than for the enzyme in the crude extract (1:2.3).

The GC chromatograms of the volatile extracts synthesized by the crude extract and the purified enzymes are documented in supplement 4.

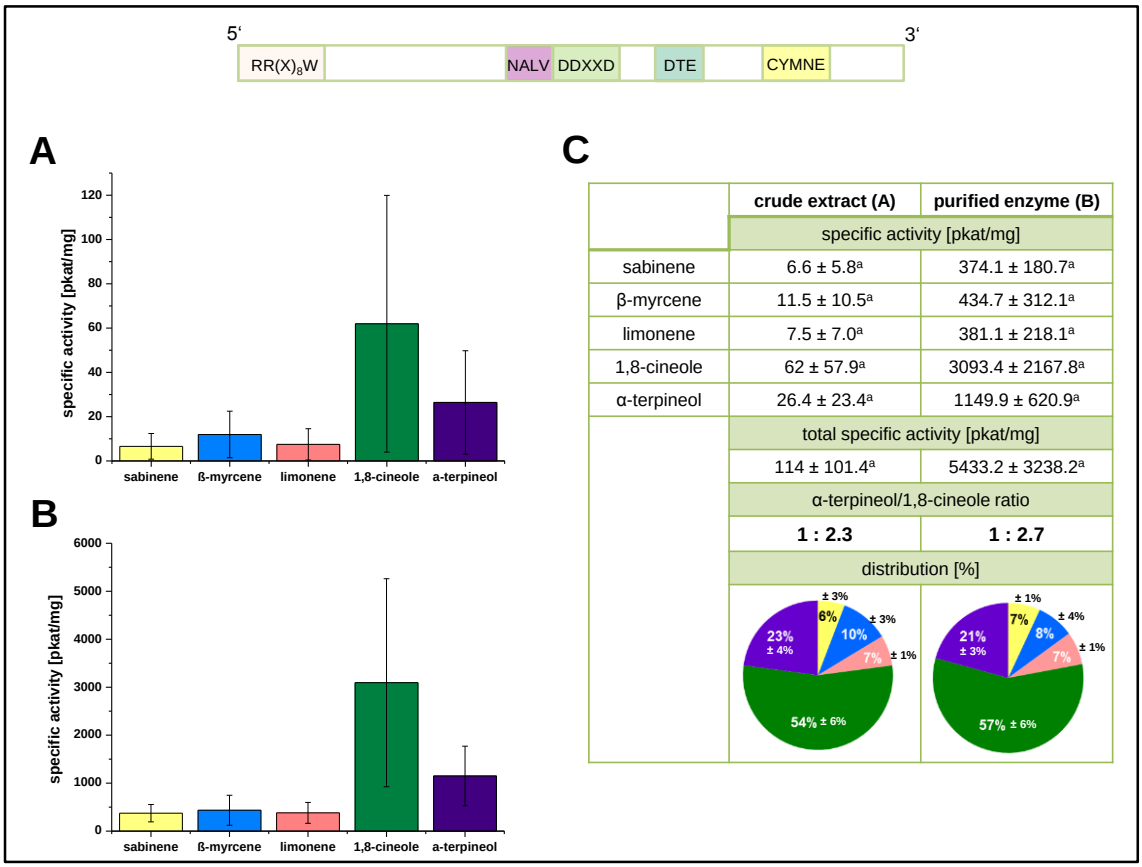


Figure 11: Analysis of the enzyme activity of the overexpressed wild type enzyme of *Nicotiana suaveolens.* A: Specific activities in pkat/mg of the crude extract (n=8) (A) and the purified enzyme (n=4) (**B)** in an after enzyme assay with GPP and 10 µg enzyme (crude extract and purified). The hexane phase of the enzyme assay was analyzed by GC-MS, and the specific activities, α-terpineol/1,8-cineole ratios and distribution (%) of volatile organic compounds for the crude extract and purified fraction are summarized in **C**. The error bars and ([a]) indicate the standard deviation.

3.3 Mutations at the N-terminal end of the 1,8-cineole synthase from *N. suaveolens*

3.3.1 Mutant G7Q

The mutation G7Q is located in the conserved $RR(X)_8$ motif (Figure 10 A). GC-MS analysis the products of this mutant resulted in the detection of five terpene compounds: sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol (for chromatograms, see supplement 4). The total specific activity of the mutant enzyme was threefold higher (406.1 pkat/mg) than that of the wild type enzyme (Figure 12 A and B). However, 1,8-cineole was the major product, with a specific activity of 206 pkat/mg, whereas the specific activities for the formation of other products were 101 pkat/mg for α-terpineol, 49 pkat/mg for sabinene,

27 pkat/mg for β-myrcene and 23 pkat/mg for limonene. No dramatic change in the product distribution compared to wild type was observed. The α-terpineol/1,8-cineole ratio was slightly reduced in the G7Q mutant (1:2.0).

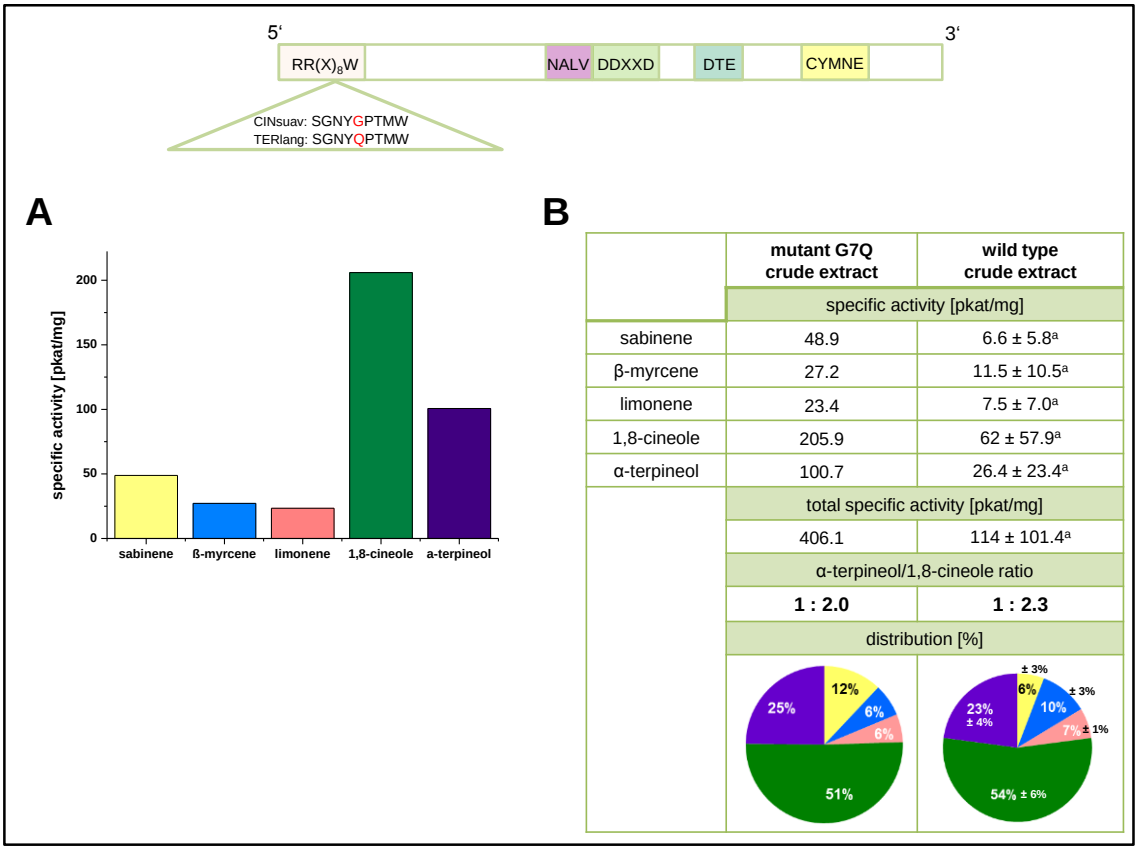


Figure 12: Analysis of the enzyme activity of the overexpressed mutant G7Q of the 1,8-cineole synthase of *N. suaveolens* (n=1). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant G7Q and the wild type. The values ± [a] indicate the standard deviation.

3.3.2 Mutant G7Q/M290L

The mutations G7Q and M290L are located N-terminal to the DDXXD motif in the RR(X)$_8$W motif and C-terminal to the DDXXD motif, with a single base mutation at position 290. The specific activities of the double mutant were reduced approximately twofold compared to the single mutant G7Q (Figure 13 vs. Figure 12). GC-MS analysis of the products formed by G7Q/M290 L revealed sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol. Figure 13 (A and B) demonstrates a specific activity of 81 pkat/mg for 1,8-cineole, which

remained the major products. The specific activities for the synthesis of the other four compounds were 39.8 pkat/mg for α-terpineol, 14 pkat/mg for β-myrcene, 13.5 pkat/mg for sabinene and only 10.3 pkat/mg for limonene. The distribution (%) of the volatiles was identical to that of wild type, and the α-terpineol/1,8-cineole ratio, which indicates the efficiency of the cyclization reaction, was identical for the double mutant G7Q/M290L and the single mutant G7Q (1:2.0).

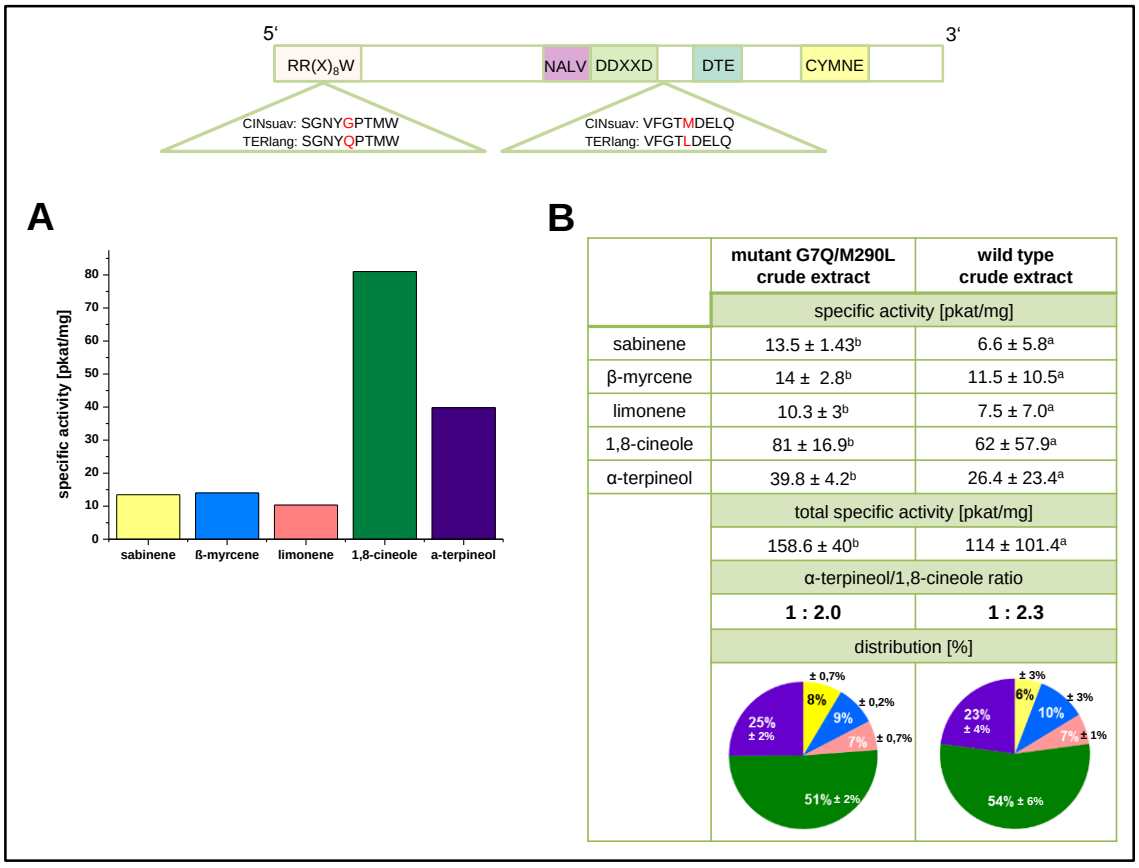


Figure 13: Analysis of the enzyme activity of the overexpressed mutant G7Q/M290L of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the positions where the mutation were introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant G7Q/M290L and the wild type. The values ± [a] indicate the standard deviation and ± [b] indicate the mean deviation.

Thus, the mutations G7Q and G7Q/M290L did not alter the α-terpineol/1,8-cineole ratios compared to the wild type enzyme. However, the production of 1,8-cineole and α-terpineol was at least twofold greater than that of the minor

products (sabinene, β-myrcene and limonene), consistent with the product distribution of the wild type enzyme.

3.4 Mutations in the middle part of the 1,8-cineole synthase of *N. suaveolens*

3.4.1 Mutant C235G

The mutation C235G is located N-terminal to the DDXXD motif, between the RWW and RDR motifs, and near two tryptophans in the 3D structure (Figure 10 B). The hypothesized influence of this mutation on the cyclization reaction could not be confirmed. The results summarized in Figure 14 (B) demonstrate that the volatile distribution (%) of this mutant was identical to that of the wild type enzyme. The α-terpineol/1,8-cineole ratio was 1:2.4 for the mutant C235G and 1:2.3 for the wild type enzyme. However, the absolute values for the specific activity of the five synthesized compounds (sabinene, β-myrcene, limonene 1,8-cineole and α-terpineol) were increased, and thus the mutation improved the enzyme activity (Figure 14 A). The specific activity for 1,8-cineole, the major product, was 287.4 pkat/mg, followed by 121 pkat/mg for α-terpineol. The specific activities for the minor products β-myrcene (54 pkat/mg), limonene (43 pkat/mg) and sabinene (33.5 pkat/mg) were higher than those of the wild type enzyme as well as the total specific activity of the mutant enzyme C235G (539.6 pkat/mg). The GC-MS analyses and chromatograms are provided in supplement 4.

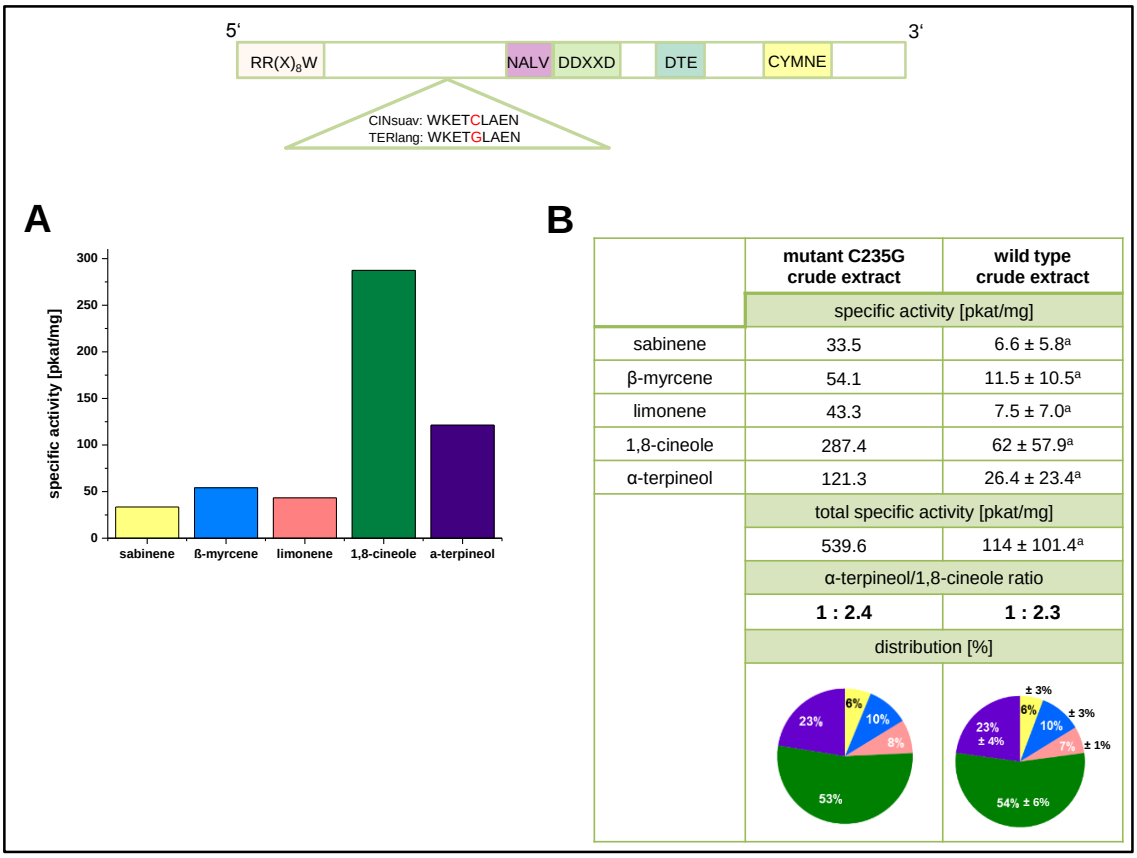


Figure 14: Analysis of the enzyme activity of the overexpressed mutant C235G of the 1,8-cineole synthase of *N. suaveolens* (n=1). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant C235G and the wild type. The values ± [a] indicate the standard deviation.

3.4.2 M290L

The single base mutation at position 290 (M290L) was localized five amino acids downstream the DDXXD motif. Figure 15 (A) shows that the synthesis of α-terpineol and 1,8-cineole benefited from this mutation because the specific activity was three times higher compared to the wild type and accounted for 79.4 pkat/mg for α-terpineol and 183 pkat/mg for 1,8-cineole (figure 15 B). The specific activities of the minor products (sabinene: 21 pkat/mg, β-myrcene: 38 pkat/mg and limonene: 21 pkat/mg) were also increased in comparison to the wild type enzyme. However, the results also showed that 1,8-cineole was the major compound in the product profile (chromatograms see supplement 4). The α-terpineol / 1,8-cineole was not altered compared to the wild type enzyme and persisted 1 : 2.3. The relative distribution (%) of the volatiles also did not

really show an alteration (figure 15 B). Despite the fact of a higher total specific activity of 344.4 pkat/mg compared to the total specific activity of the wild type enzyme.

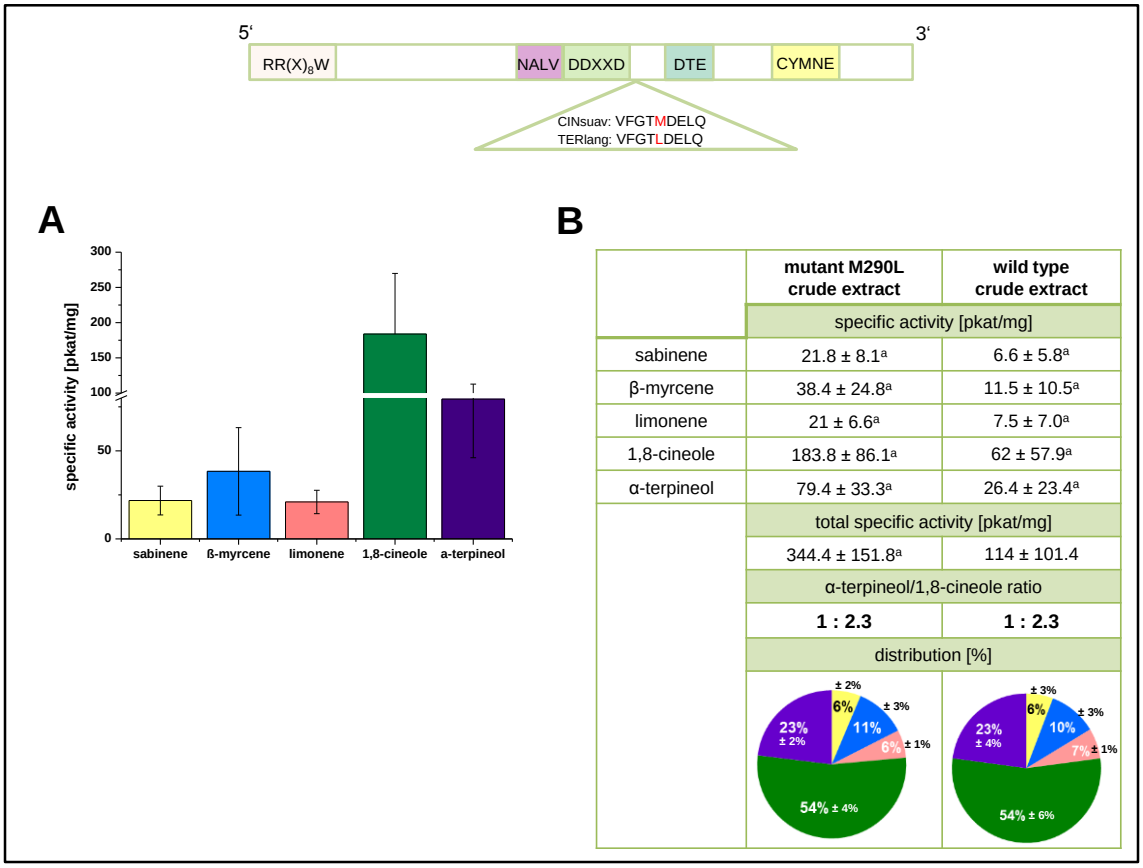


Figure 15: Analysis of the enzyme activity of the overexpressed mutant M290L of the 1,8-cineole synthase of *N. suaveolens* (n=3). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant M290L and the wild type. The error bars indicate the standard deviation. The values ± [a] indicate the standard deviation.

3.4.3 Mutant H298D

The mutation H298D is located C-terminal to the DDXXD motif. The results of two experiments were inconsistent and are presented in Figure 16. In the first experiment, only α-terpineol and 1,8-cineole could be detected. Both specific activities were lower than those of the wild type enzyme: 16.6 pkat/mg for 1,8-cineole and 4.1 pkat/mg for α-terpineol. The α-terpineol/1,8-cineole ratio was 1;4, a twofold increase compared to the wild type enzyme. The volatile

distribution (%) was 80 % 1,8-cineole and 20 % α-terpineol. In the second experiment, the typical five monoterpenoids sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol were observed (Figure 16 and supplement 4). The total specific activity of the mutated enzyme increased compared to the wild type enzyme, to 567.5 pkat/mg. Values of 344.1 pkat/mg for 1,8-cineole as the major compound, 139.2 pkat/mg for α-terpineol, 33.2 pkat/mg for limonene, 27.8 pkat/mg for sabinene and 23.2 pkat/mg for β-myrcene were observed. The distribution of the volatiles (%) was altered compared to the wild type enzyme: 61 % 1,8-cineole and 25 % α-terpineol. The proportion of β-myrcene increased twofold compared to the wild type enzyme. The α-terpineol/1,8-cineole ratio in the second experiment was 1:2.5, similar to that of the wild type enzyme.

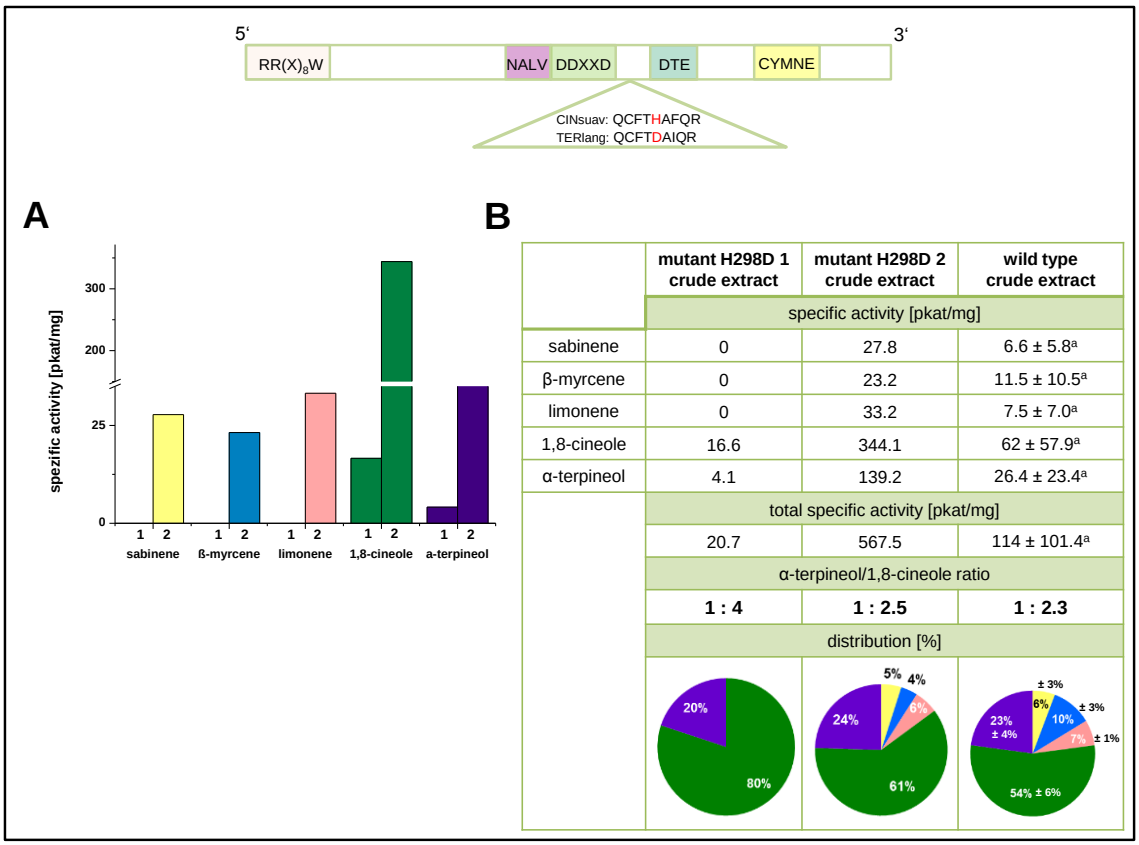


Figure 16: Analysis of the enzyme activity of the overexpressed mutant H298D of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant H298D and the wild type. The values ± [a] indicate the standard deviation.

3.4.4 Mutant M290L/H298D

The combination of two single base mutations, M290L and H298D, produced unexpected results (Figure 17 A and B). First, different product outcomes were observed in two experiments. Second, both experiments indicated a decrease in specific activity compared to the wild type enzyme. In the first experiment, four compounds (β-myrcene, limonene, 1,8-cineole and α-terpineol; see supplement 4) were detected, whereas in the second experiment, only 1,8-cineole and α-terpineol were observed. In the two experiments, the specific activity was 40.9 pkat/mg and 13 pkat/mg for 1,8-cineole and 20 pkat/mg and 8 pkat/mg for α-terpineol. Sabinene was not synthesized by the M290L/H298D mutant. The specific activities for β-myrcene and limonene were 7 pkat/mg and 1 pkat/mg, respectively (Figure 17, B). The circle diagram (Figure 17) revealed a different distribution of α-terpineol and 1,8-cineole compared to the wild type enzyme. The rate for α-terpineol was slightly decreased to 29 % in the first experiment and to 39 % in the second experiment, whereas the rate for β-myrcene was comparable to that of wild type (10 %). The major compound in both experiments was 1,8-cineole, at 58 % and 61 %. Thus, the double mutation M290L/H298D altered the product profile and total specific activity as well as the α-terpineol/1,8-cineole ratio compared to the wild type enzyme.

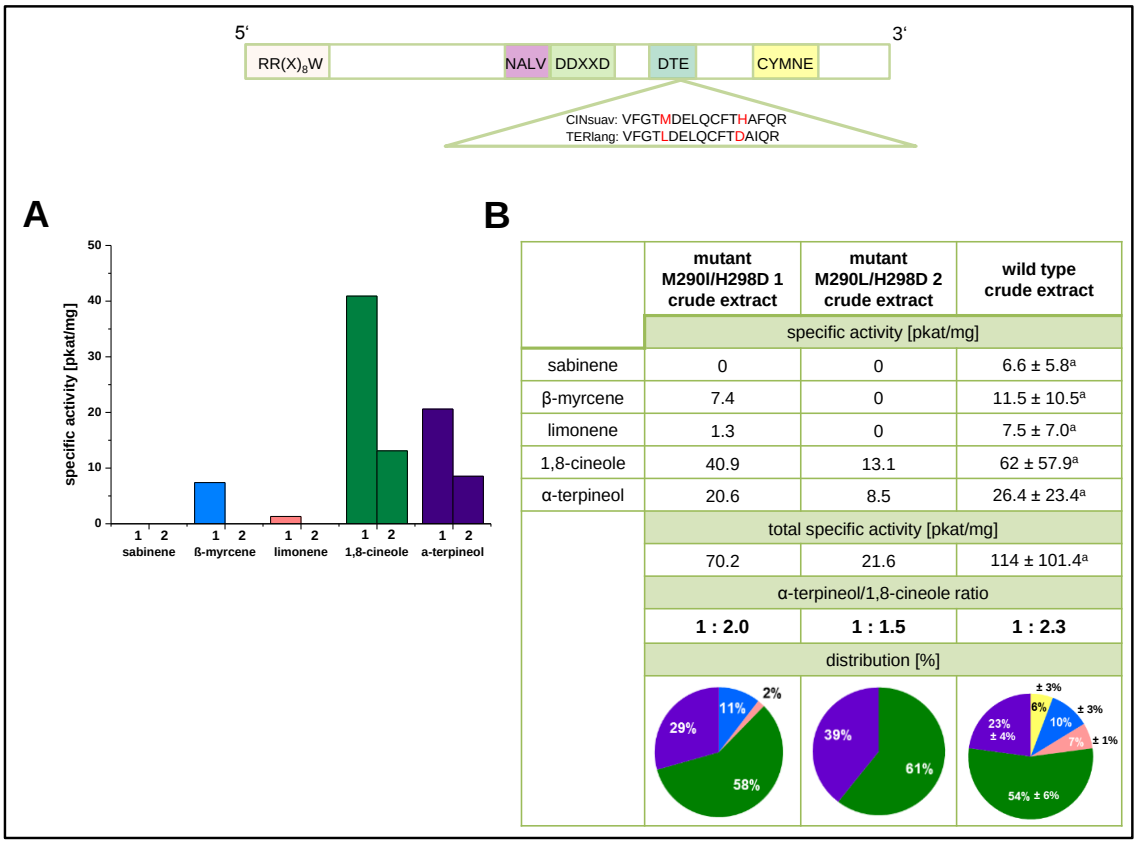
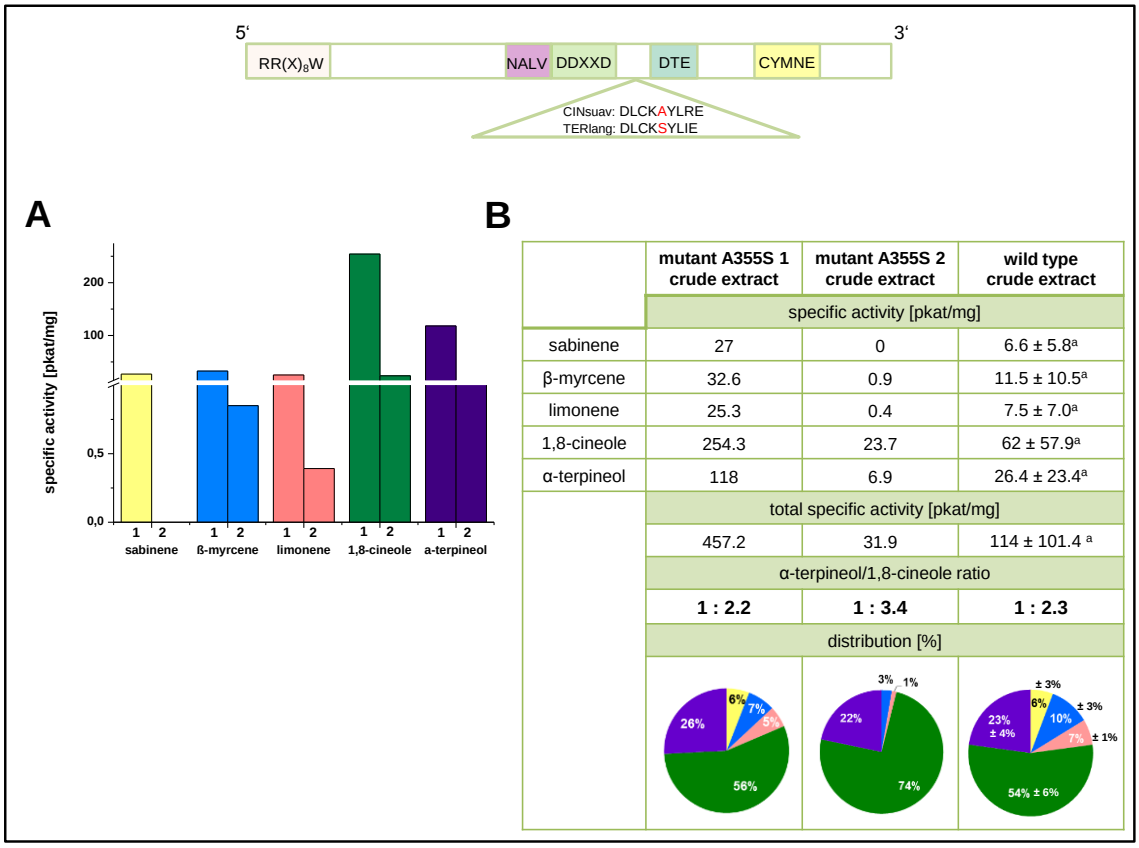


Figure 17: Analysis of the enzyme activity of the overexpressed mutant M290L/H298D of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the positions where the mutations were introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant M290L/H298D and the wild type. The values ± [a] indicate the standard deviation.

3.4.5 Mutant A355S

In the 3D model, the amino acid at position 355 is in close proximity to the DDXXD motif on a different helix (Figure 10). The mutation A355S was expected to influence the reaction mechanism because of the proximity of the co-factor binding motif. Figure 18 (A and B) shows that the total specific activity determined in the first experiment was higher (457.2 pkat/mg) than that of the wild type enzyme. The bicyclic monoterpene 1,8-cineole was the major compound and was synthesized with a specific activity of 254.3 pkat/mg, while the specific activities for the other products were 118 pkat/mg for α-terpineol, 32.6 pkat.mg for β-myrcene, 27 pkat/mg for sabinene and 25.3 pkat/mg for limonene. The volatile distribution (%) and the α-terpineol/1,8-cineole ratio (1:2.2) of the mutant A355S were comparable to those of the wild type enzyme.

A different product outcome was observed in the second experiment (Figure 18 and supplement 4), with decreased activities for the four detected monoterpenoid compounds: α-terpineol (7 pkat/mg), 1,8-cineole (23.7 pkat/mg), limonene (0.4 pkat/mg) and β-myrcene (0.9 pkat/mg) (Figure 18 A and B). The by-product sabinene was not detected. The distribution was shifted and reached 74 % 1,8-cineole. The proportion of α-terpineol (22 %) was identical to that produced by the wild type enzyme. The proportions of limonene and β-myrcene decreased to 1 % and 3 %, respectively. The efficiency of the cyclization reaction increased to 1:3.4. The mutation A355S may alter this ratio due to its close proximity to the important DDXXD motif.



	mutant A355S 1 crude extract	mutant A355S 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	27	0	6.6 ± 5.8[a]
β-myrcene	32.6	0.9	11.5 ± 10.5[a]
limonene	25.3	0.4	7.5 ± 7.0[a]
1,8-cineole	254.3	23.7	62 ± 57.9[a]
α-terpineol	118	6.9	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	457.2	31.9	114 ± 101.4 [a]
	α-terpineol/1,8-cineole ratio		
	1 : 2.2	**1 : 3.4**	**1 : 2.3**
	distribution [%]		

Figure 18: Analysis of the enzyme activity of the overexpressed mutant A355S of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant A355S and the wild type. The values ± [a] indicate the standard deviation.

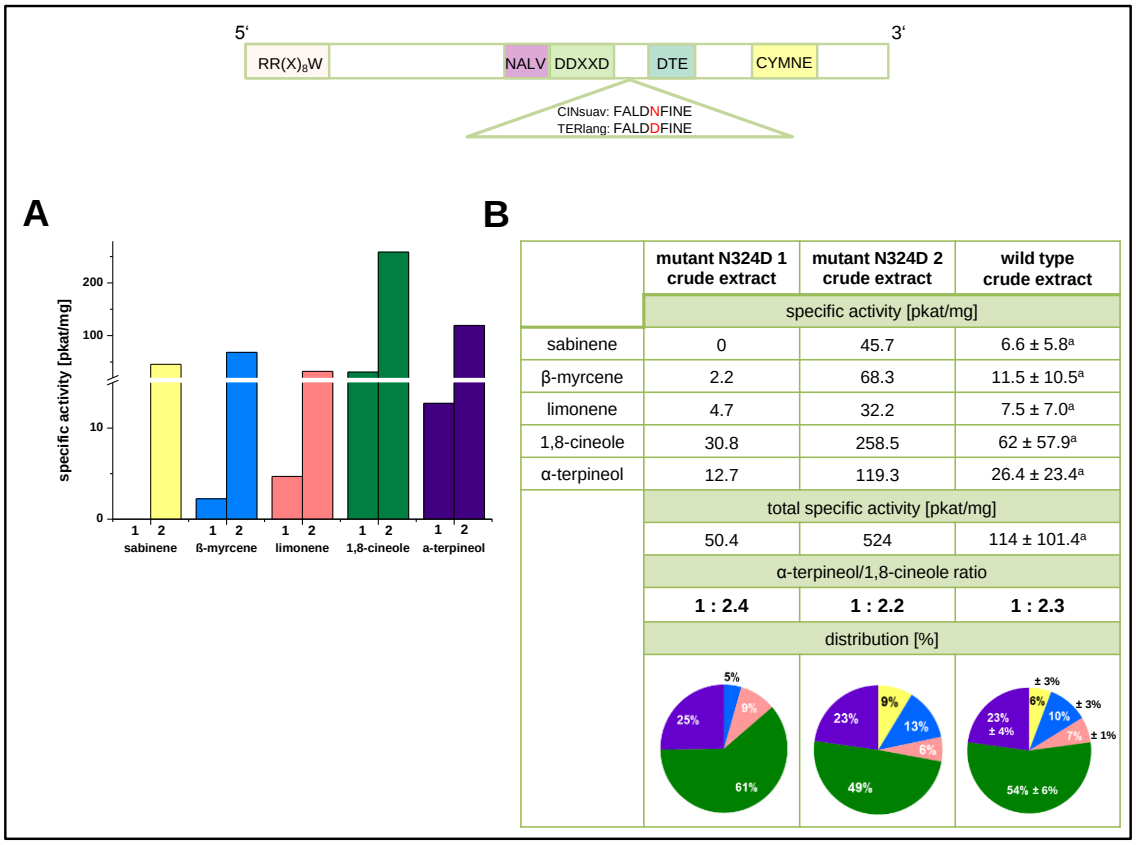
3.4.6 Mutant F300I

The single base mutation F300I is C-terminal to the DDXXD motif. While this mutant could be isolated and characterized by SDS-PAGE and Western blot, no monoterpenoid products were detectable by GC-MS analysis (for SDS-PAGE and chromatogram, see supplement 4). These results indicate that phenylalanine 300 has an important impact on the structure and, consequently, the functionality of the enzyme.

3.4.7 Mutant N324D

The enzyme containing the mutation N324D (Figure 19 A and B), which is C-terminal to the DDXXD motif, exhibited higher specific activities in one experiment compared to wild type. All five monoterpenoid compounds except sabinene were detected in experiment 1. The specific activities of the mutant enzyme N324D in experiment one were 30.8 pkat/mg for 1,8-cineole, 12.7 pkat/mg for α-terpineol, 4.7 pkat/mg for limonene and only 2.2 pkat/mg for β-myrcene. The α-terpineol/1,8-cineole ratio was 1:2.4 comparable, to the wild type enzyme. Compared to the product distribution of the wild type enzyme, 1,8-cineole increased to 61 %, α-terpineol to 25 % and limonene to 9 %, whereas β-myrcene decreased by half (Figure 19 B). The results of the second overexpression experiment with mutant enzyme N324D revealed increased specific activities for all five compounds: 1,8-cineole (258.5 pkat/mg), α-terpineol (119.3 pkat/mg), β-myrcene (68.3 pkat/mg), sabinene (45.7 pkat/mg) and limonene (32.2 pkat/mg). The α-terpineol/1,8-cineole ratio was 1:2.2 and was therefore not altered compared to the wild type enzyme. As shown in the distribution chart, the proportion of 1,8-cineole decreased slightly to 49 %, while the proportions of β-myrcene and sabinene increased to 13 % and 9 %, respectively. The proportions of α-terpineol and limonene were comparable to those of wild type. The N324D mutant exhibited decreased or increased total specific activities of 50.4 pkat/mg and 524 pkat/mg, respectively, in the two experiments. However, these differences in specific activity did not have a great

impact on the relative fractions and therefore apparently no influence on the function of the enzyme, e.g., 1,8-cineole remained the major compound.



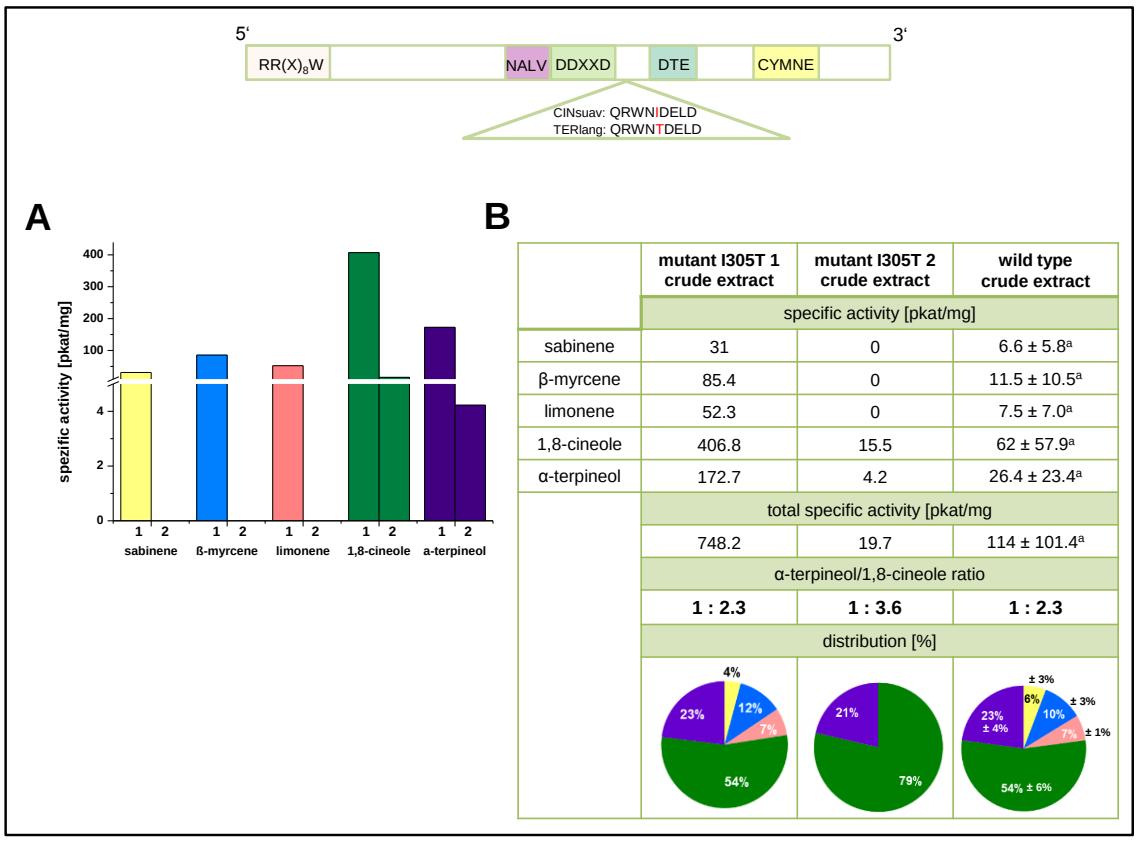
	mutant N324D 1 crude extract	mutant N324D 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	0	45.7	6.6 ± 5.8[a]
β-myrcene	2.2	68.3	11.5 ± 10.5[a]
limonene	4.7	32.2	7.5 ± 7.0[a]
1,8-cineole	30.8	258.5	62 ± 57.9[a]
α-terpineol	12.7	119.3	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	50.4	524	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 2.4	**1 : 2.2**	**1 : 2.3**
	distribution [%]		

Figure 19: Analysis of the enzyme activity of the overexpressed mutant N324D of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant N324D and the wild type. The values ± [a] indicate the standard deviation.

3.4.8 Mutant I305T

The two experiments with the mutant I305T produced results similar to those obtained with the mutant H298D. Two or five monoterpenoid products were observed in the two experiments (Figure 20 and chromatogram in supplement 4). In the first experiment, the specific activities of the enzyme for each synthesized compound were increased compared to the wild type enzyme, reaching values of 406.8 pkat/mg for 1,8-cineole, 172.7 pkat/mg for α-terpineol, 85.4 pkat/mg for β-myrcene, 52.3 pkat/mg for limonene and 31 pkat/mg for sabinene. The specific activity of the mutant enzyme I305T for 1,8-cineole was

the highest observed among all mutants and products. However, the volatile distribution was not substantially altered compared to the wild type enzyme (Figure 20, B), and the α-terpineol/1,8-cineole ratio was 1:2.3. The total specific activity of the mutant enzyme in the first experiment (748.2 pkat/mg) was sixfold higher than that of the wild type enzyme. The specific activities measured in experiment two were tremendously reduced, with values of 15.5 pkat/mg for 1,8-cineole and 4.2 pkat/mg for α-terpineol (Figure 20 B). Consequently, the α-terpineol/1,8-cineole ratio increased to 1:3.6, indicating that the efficiency of the cyclization reaction toward 1,8-cineole was optimized. In both experiments with the mutant I305T, 1,8-cineole was the major compound, but because minor products (sabinene, β-myrcene and limonene) were not detected and the proportion of α-terpineol remained constant, the contribution of 1,8-cineole increased to 79 % in the second experiment.

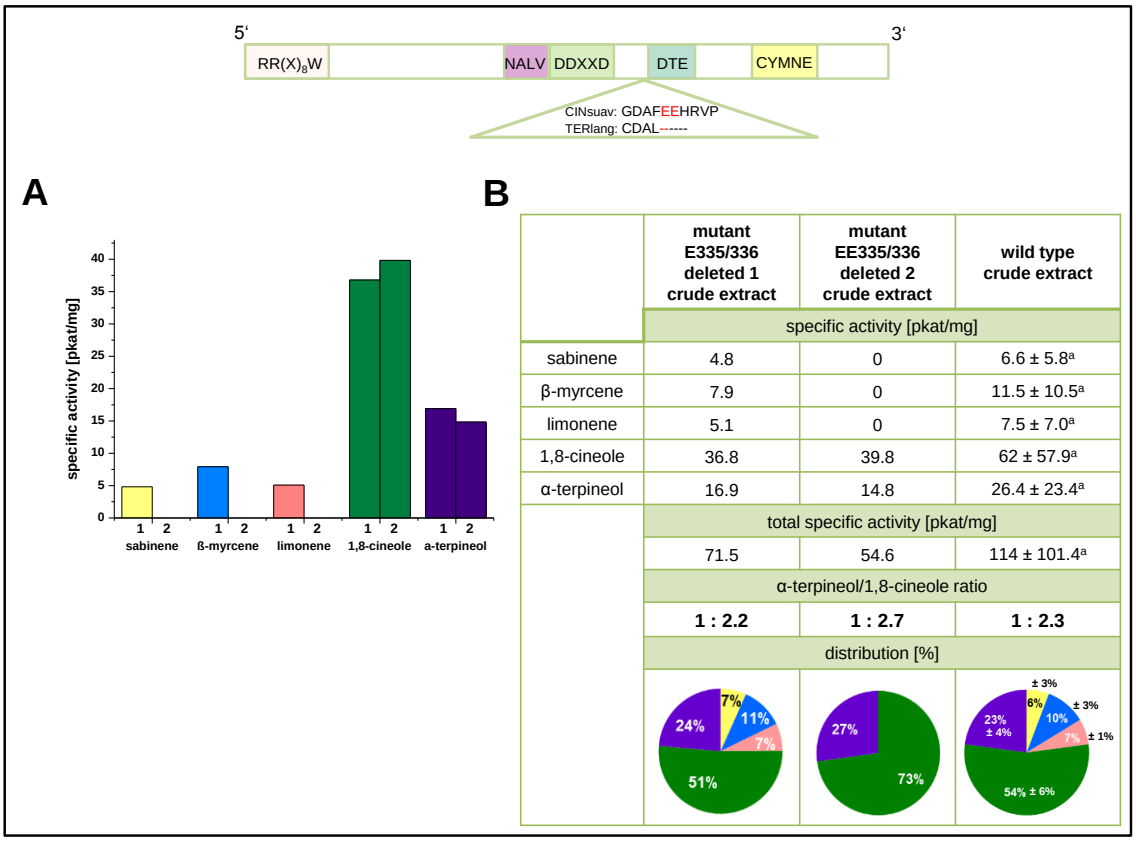


	mutant I305T 1 crude extract	mutant I305T 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	31	0	6.6 ± 5.8[a]
β-myrcene	85.4	0	11.5 ± 10.5[a]
limonene	52.3	0	7.5 ± 7.0[a]
1,8-cineole	406.8	15.5	62 ± 57.9[a]
α-terpineol	172.7	4.2	26.4 ± 23.4[a]
	total specific activity [pkat/mg		
	748.2	19.7	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 2.3	**1 : 3.6**	**1 : 2.3**
	distribution [%]		

Figure 20: Analysis of the enzyme activity of the overexpressed mutant I305T of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant I305T and the wild type. The values ± [a] indicate the standard deviation.

3.4.9 Mutant EE335/336-deleted

The two glutamates at positions 335 and 336 of the 1,8-cineole synthase of N*. suaveolens* were expected to play a role in the cyclization reaction to 1,8-cineole because they were missing in the sequence of the α-terpineol synthase of *N. langsdorfii*. As shown in Figure 21 (A and B), different results were obtained in two experiments (chromatograms in supplement 4), as observed in the analyses of the other mutants, such as I305T. In both experiments, the specific activity of the deletion mutant EE335/336 was lower than that of the wild type enzyme, and the values for 1,8-cineole and α-terpineol in both experiments were approximately the same (37-40 pkat/mg for 1,8-cineole and 15-17 pkat/mg for α-terpineol). The specific activities for the minor products in the first experiment were 8 pkat/mg for β-myrcene, 5 pkat/mg for limonene and 5 pkat/mg for sabinene (Figure 21 A and B). The distribution chart in the first experiment was not altered from that of wild type, while in the second experiment, the relative contributions of 1,8-cineole and α-terpineol were 73 % and 27 %, respectively. The α-terpineol/1,8-cineole ratio was slightly lower in the first experiment (1:2.2) and increased in the second experiment (1:2.7). These results indicate that deletion of EE335/336 decreased the total specific activity of the enzyme, reducing the synthesis of monoterpenoid compounds.



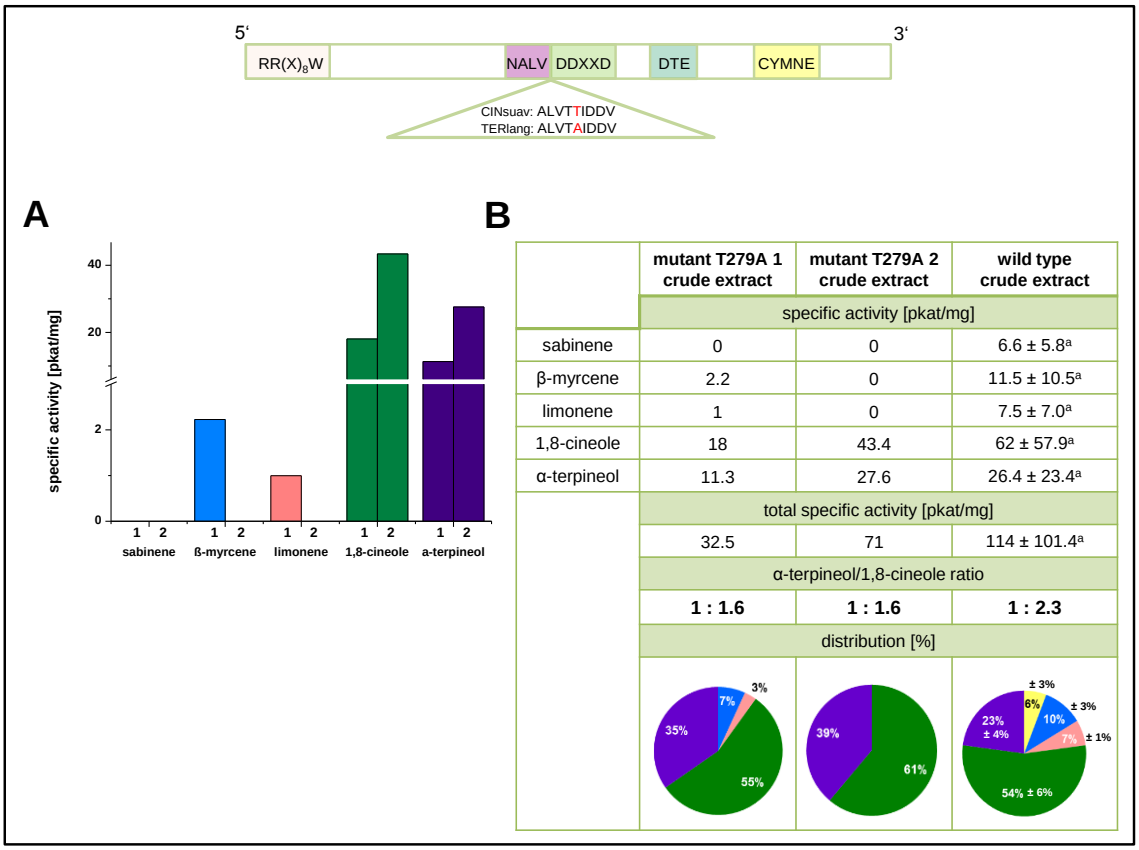
	mutant E335/336 deleted 1 crude extract	mutant EE335/336 deleted 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	4.8	0	6.6 ± 5.8[a]
β-myrcene	7.9	0	11.5 ± 10.5[a]
limonene	5.1	0	7.5 ± 7.0[a]
1,8-cineole	36.8	39.8	62 ± 57.9[a]
α-terpineol	16.9	14.8	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	71.5	54.6	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 2.2	**1 : 2.7**	**1 : 2.3**
	distribution [%]		

Figure 21: Analysis of the enzyme activity of the overexpressed mutant EE335/336-deleted of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the positions where the mutations were introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant EE335/336-deleted and the wild type. The values ± [a] indicate the standard deviation.

3.4.10 Mutant T279A

The single-base mutation T279A is located between the NALV motif and the DDXXD motif. This mutant was generated because the substitution alters the properties of the amino acid and is in close proximity to the important DDXXD motif. Two experiments with different results were obtained (Figure 22 A and B). 1,8-Cineole and α-terpineol were detected in both experiments, while β-myrcene and limonene were only detected in the first experiment (for chromatogram, see supplement 4). Sabinene was absent in both experiments. The total specific activity of the mutant T279A was reduced (32.5 pkat/mg and 71 pkat/mg, respectively) in both experiments compared to the wild type enzyme. The values for 1,8-cineole were 18 pkat/mg in the first experiment and more than doubled in the second experiment (43 pkat/mg). The specific activity

for α-terpineol was 11 pkat/mg in the first experiment and 27 pkat/mg in the second experiment. The minor products detected in the second experiment, β-myrcene and limonene, were synthesized with a specific activity of 2 pkat/mg and 1 pkat/mg. In both experiments, α-terpineol reached proportions of 35-39 %, higher than that of the wild type enzyme (Figure 22 B). The proportion of 1,8-cineole was 61 % and 55 %, similar to that observed for the wild type enzyme. Compared to wild type, the proportions of β-myrcene and limonene decreased to 7 % and 3 % in the first experiment and 0 % in the second experiment. In both experiments, the α-terpineol/1,8-cineole ratio was 1:1.6 and significantly lower than that of the wild type enzyme, indicating that the efficiency of the cyclization reaction was reduced in the mutant enzyme T279A compared to the wild type enzyme.



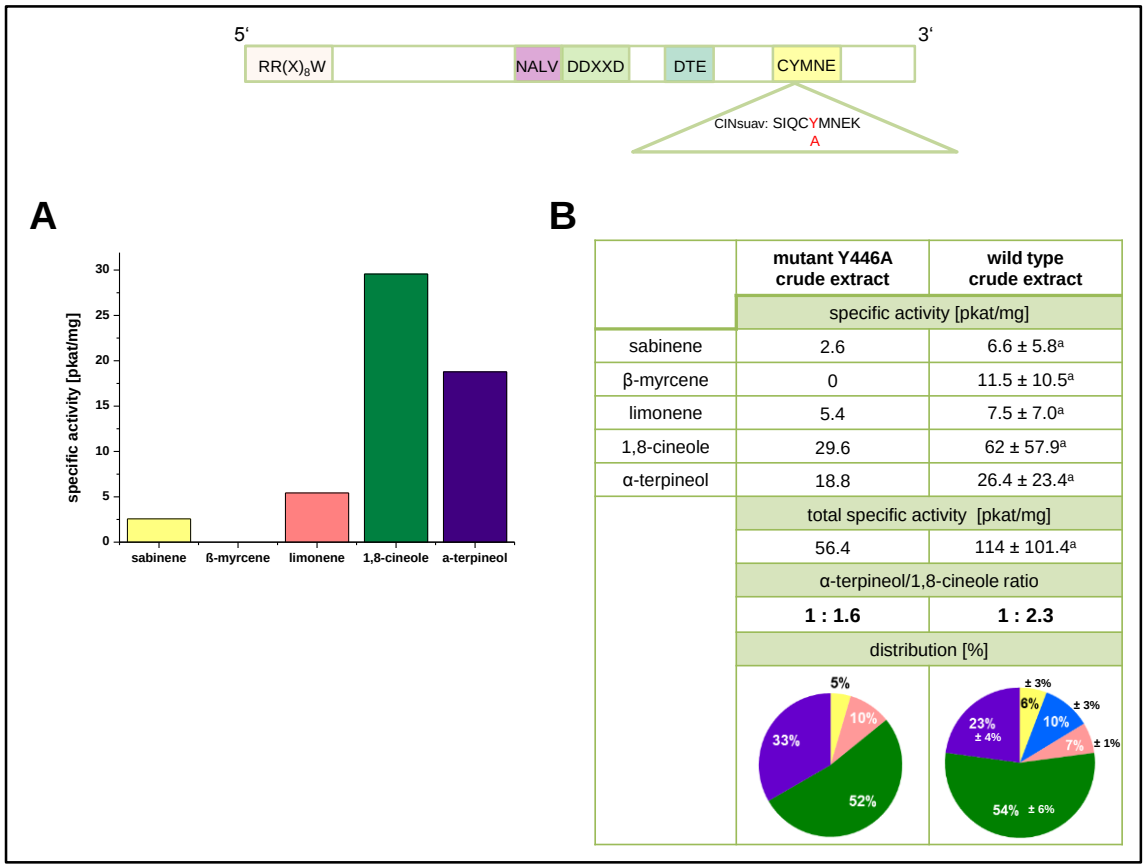
	mutant T279A 1 crude extract	mutant T279A 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	0	0	6.6 ± 5.8[a]
β-myrcene	2.2	0	11.5 ± 10.5[a]
limonene	1	0	7.5 ± 7.0[a]
1,8-cineole	18	43.4	62 ± 57.9[a]
α-terpineol	11.3	27.6	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	32.5	71	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 1.6	**1 : 1.6**	**1 : 2.3**
	distribution [%]		

Figure 22: Analysis of the enzyme activity of the overexpressed mutant T279A of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant T279A and the wild type. The values ± [a] indicate the standard deviation.

3.5 Mutations at the C-terminal end of the 1,8-cineole synthase of *N. suaveolens*

3.5.1 Mutant Y446A

The mutation Y446A is located in the functionally unknown CYMNE motif. This residue was hypothesized to influence the protonation of the double bond of α-terpineol to form 1,8-cineole. This protonation might be altered in the mutant because of the absence of the hydroxyl group of tyrosine. The specific activity of the overexpressed mutated enzyme Y446A (Figure 23 A and B) was lower for the four synthesized compounds (sabinene, limonene, 1,8-cineole and α-terpineol) compared to the wild type enzyme. The acyclic compound β-myrcene could not be detected (for chromatogram, see supplement 4). The specific activities of the mutant Y446A were 29.6 pkat/mg for 1,8-cineole, 18.8 pkat/mg for α-terpineol, 5.4 pkat/mg for limonene and only 2.6 pkat/mg for sabinene (Figure 23, B). Therefore, the distribution was altered compared to the wild type enzyme and reached 33 % for α-terpineol, 52 % for 1,8-cineole, 10 % for limonene and only 5 % for sabinene. The α-terpineol/1,8-cineole ratio was also reduced to 1:1.6 compared to wild type. These results indicate that the efficiency of the cyclization reaction to 1,8-cineole decreased, and the total specific activity decreased to 56.4 pkat/mg.



	mutant Y446A crude extract	wild type crude extract
	specific activity [pkat/mg]	
sabinene	2.6	6.6 ± 5.8[a]
β-myrcene	0	11.5 ± 10.5[a]
limonene	5.4	7.5 ± 7.0[a]
1,8-cineole	29.6	62 ± 57.9[a]
α-terpineol	18.8	26.4 ± 23.4[a]
	total specific activity [pkat/mg]	
	56.4	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio	
	1 : 1.6	**1 : 2.3**
	distribution [%]	

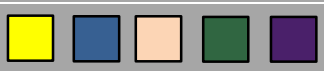
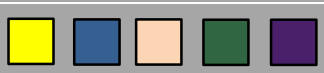
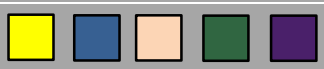
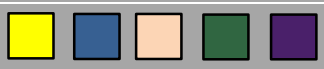
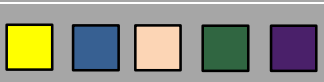
Figure 23: Analysis of the enzyme activity of the overexpressed mutant Y446A of the 1,8-cineole synthase of *N. suaveolens* (n=1). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant Y446A and the wild type. The values ± [a] indicate the standard deviation.

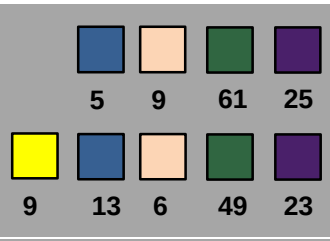
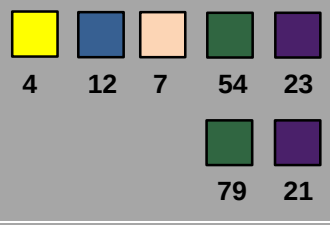
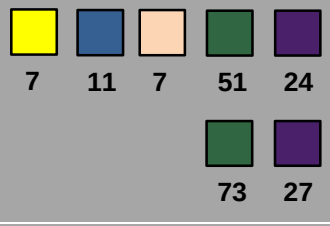
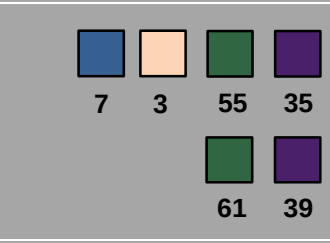
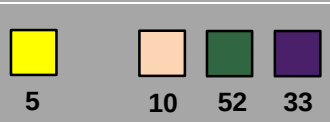
3.5.2 Mutant Y502V

The single base mutation Y502V is located in the C-terminal region. This mutant was generated because it was speculated that the hydroxyl group of tyrosine might influence the protonation of α-terpineol. Although the protein was visible by SDS-PAGE (supplement 4), GC-MS analysis (n=2) did not indicate monoterpenoid compounds in the hexane phase (for chromatograms, see supplement 4); only the internal standard, cis-nerolidol, was visible. Thus, this amino acid alteration in the C-terminal region of the enzyme, in which tyrosine was changed to the non-hydroxylated amino acid valine, resulted in an enzyme with no activity.

In summary (Table 2), none of the 14 analyzed mutant enzymes exhibited an inversion of the α-terpineol/1,8-cineole ratio compared to the wild type enzyme. For the mutants H298D (Figure 16), A355S (Figure 18) and I305T (Figure 20), the efficiency of the synthesis of 1,8-cineole increased compared to the wild type, as indicated by α-terpineol/1,8-cineole ratios of 1:4, 1:3.4 and 1:3.6, respectively. A decreased ratio of α-terpineol/1,8-cineole of 1:1.6 was detected for T279A and Y446A mutants of the 1,8-cineole synthase of *N. suaveolens*. The total specific activities of the mutant enzymes varied compared to the wild type enzyme varied greatly. The experiments for mutants G7Q, C235G, M290L, the first experiments for the mutants H298D, A355S and I305T, and the second experiment for the mutant N324D indicated significantly increased total specific activities of 344-748 pkat/mg compared to the total specific activity of the wild type enzyme (114 pkat/mg). Although there were substantial discrepancies in the absolute values of the specific activities between the mutant enzymes and the wild type enzyme, the product distributions (%) were often comparable to that of the wild type enzyme. The mutant enzymes Y446A, T279A, N324D, A355S and M290L/H298D synthesized four of the five compounds synthesized by the wild type enzyme. GC-MS analysis of the second experiment for the mutants H298D, I305T, EE335/336-deleted, T279A and M290L/H298D detected only two monoterpenoid compounds, resulting in an altered distribution. The mutant enzymes F300I and Y502V were unable to synthesize any monoterpenoid compounds. The influence of mutations near the important DDXXD motif (Figure 10 B) on enzyme activity and product outcome cannot be predicted. Mutations distant as well as close to the DDXXD motif could affect the total specific activity of the mutant enzyme. The analysis of the mutant enzymes EE335/336-deleted and Y446A revealed decreased specific activity, even though these mutations are distant from the DDXXD motif. By contrast, mutations closer to the DDXXD motif (e.g., M290L, H298D, and A355S, Figure 10 B) increased the specific activity of the enzyme.

Table 2: Summary of the results of 14 mutants. The abbreviation of the mutation, the α-terpineol / 1,8-cineole ratios of the first and second experiments of the mutated enzymes, the total specific activity and the volatile distribution numbers below colored squares are listed and compared to the wild type crude extract. Color code, yellow: sabinene, blue: β-myrcene, wheat: limonene, green: 1,8-cineole and purple: α-terpineol.

	α-terpineol/ 1,8-cineole ratio (wild type enzyme crude extract: 1:2.3)	**total specific activity [pkat/mg] (wild type enzyme crude extract: 114 ± 101.4 pkat/mg)**	**distribution [%] wild type enzyme crude extract:**  **6 10 7 54 23**
G7Q	1:2.0	406.1	 **12 6 6 51 25**
G7Q/M290L	1:2.0	158.6 ± 40	 **8 9 7 51 25**
C235G	1:2.4	539.6	 **6 10 8 53 23**
M290L	1:2.3	344.4 ± 151.8	 **6 11 6 54 23**
H298D			
1st	1:4.0	20.7	 **80 20**
2nd	1:2.5	567.5	 **5 4 6 61 24**
M290L/H298D			
1st	1:2.0	70.2	 **11 2 58 29**
2nd	1:1.5	21.6	 **61 39**
A355S			
1st	1:2.2	457.2	 **6 7 5 56 26**
2nd	1:3.4	31.9	 **3 1 74 22**

N324D 1st 2nd	 1:2.4 1:2.1	 50.4 524.0	
I305T 1st 2nd	 1:2.3 1:3.6	 748.2 19.7	
EE335/336-deleted 1st 2nd	 1:2.2 1:2.7	 71.5 54.6	
T279A 1st 2nd	 1:1.6 1:1.6	 32.5 71.0	
Y446A	1:1.6	56.4	
F300I	0:0	0	
Y502V	0:0	0	

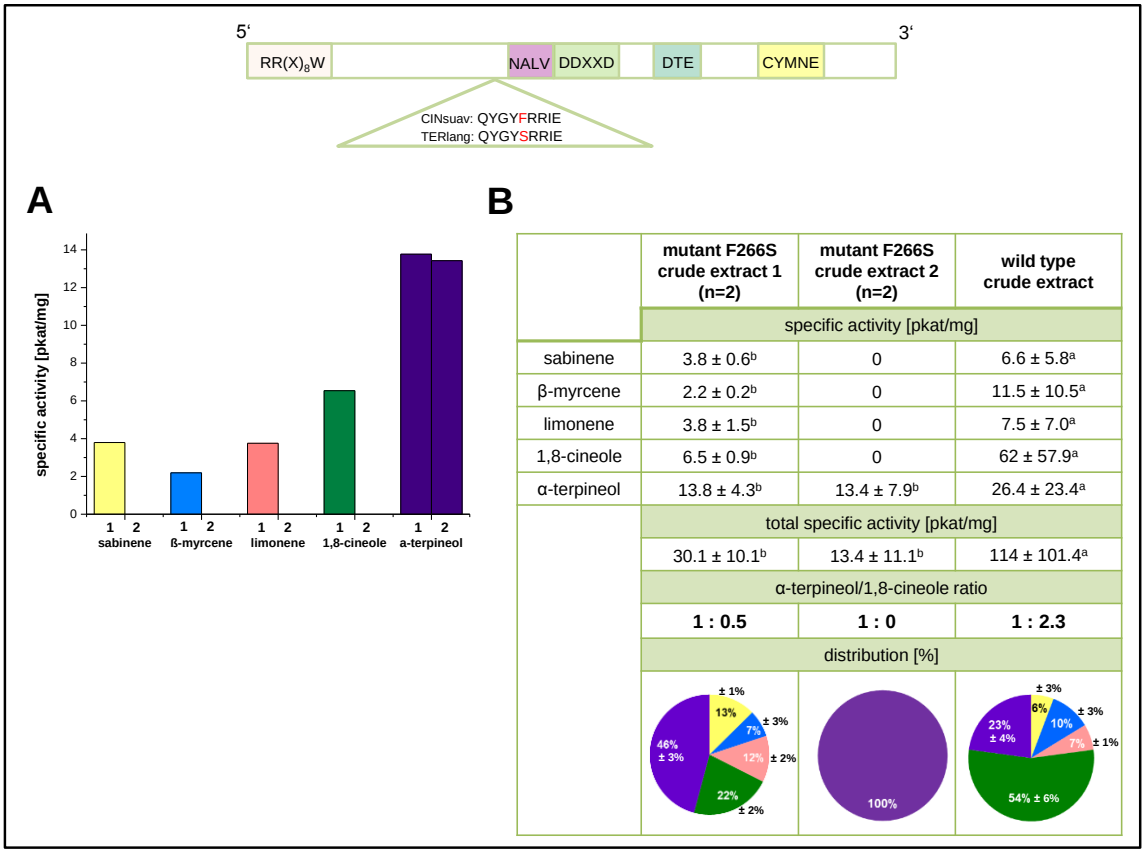
3.6 Single base mutations at position 266 in the 1,8-cineole synthase gene of *N. suaveolens*

The amino acid at position 266 was of specific interest because it is N-terminal to the NALV and DDXXD motifs. The 3D protein model (Figure 10) indicates that this amino acid is positioned at the other end of the same helix as the DDXXD motif. Therefore, this position was studied in more detail. Five mutants were constructed: F266S, F266T, F266V, F266Y and F266C. These mutations were selected to evaluate the influence of steric alterations and side chain changes.

3.6.1 Mutant F266S

The GC-MS analysis of the mutant enzyme F266S in the crude extract revealed (Figure 24 A and B) that the product outcome was altered compared to wild type. In first experiment (n=2), five monoterpenoid compounds were detected, whereas in the second experiment (n=2), only α-terpineol was detected (for chromatograms, see supplement 4). Both analyses indicated that α-terpineol was the main product, while the major product of the wild type enzyme in the crude extract was 1,8-cineole. The specific activities of the F266S enzyme were lower than those of wild type: 7 pkat/mg for 1,8-cineole, 14 pkat/mg for α-terpineol, 4 pkat/mg for limonene and sabinene and only 2 pkat/mg for β-myrcene (Figure 24, A). In the first experiment, a reduced proportion of 1,8-cineole (22 %) was observed, whereas the α-terpineol value increased to 46 %. These experiments were the first to demonstrate a significant increase in the proportion of α-terpineol compared to the wild type enzyme. These elevated levels of α-terpineol were confirmed in the second experiment, in which only α-terpineol was detected. The distribution of the synthesized minor products (sabinene: 13 %, β-myrcene: 7 %, and limonene: 12 %) was approximately the same as that observed for the wild type enzyme. Consequently, the α-terpineol/1,8-cineole ratio was 1:0.5 in the first experiment and 1:0 in the second experiment (Figure 24, B). Thus, the mutant F266S exhibited a switch in the α-terpineol/1,8-cineole ratio compared to the wild type enzyme. More α-

terpineol was produced than 1,8-cineole when the bulky amino acid phenylalanine was substituted by the small amino acid serine, which features a hydroxyl group in its side chain.



	mutant F266S crude extract 1 (n=2)	mutant F266S crude extract 2 (n=2)	wild type crude extract
	specific activity [pkat/mg]		
sabinene	3.8 ± 0.6[b]	0	6.6 ± 5.8[a]
β-myrcene	2.2 ± 0.2[b]	0	11.5 ± 10.5[a]
limonene	3.8 ± 1.5[b]	0	7.5 ± 7.0[a]
1,8-cineole	6.5 ± 0.9[b]	0	62 ± 57.9[a]
α-terpineol	13.8 ± 4.3[b]	13.4 ± 7.9[b]	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	30.1 ± 10.1[b]	13.4 ± 11.1[b]	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 0.5	**1 : 0**	**1 : 2.3**
	distribution [%]		

Figure 24: Analysis of the enzyme activity of the overexpressed mutant F266S of the 1,8-cineole synthase of *N. suaveolens* (n=4). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant F266S and the wild type. The values ± [a] indicate the standard deviation and ± [b] indicate the mean deviation.

3.6.2 Mutant F266T

Because of the promising results obtained with the F266S mutant enzyme and the potential role of the hydroxyl group in the side chain, the mutants F266T and F266Y were generated. Only α-terpineol was detected when the F266T enzyme was analyzed. The specific activity was 15 pkat/mg, lower than that of the wild type enzyme (Figure 25 A and B). The α-terpineol/1,8-cineole ratio was 1:0, indicating that the cyclization reaction to 1,8-cineole was completely abolished.

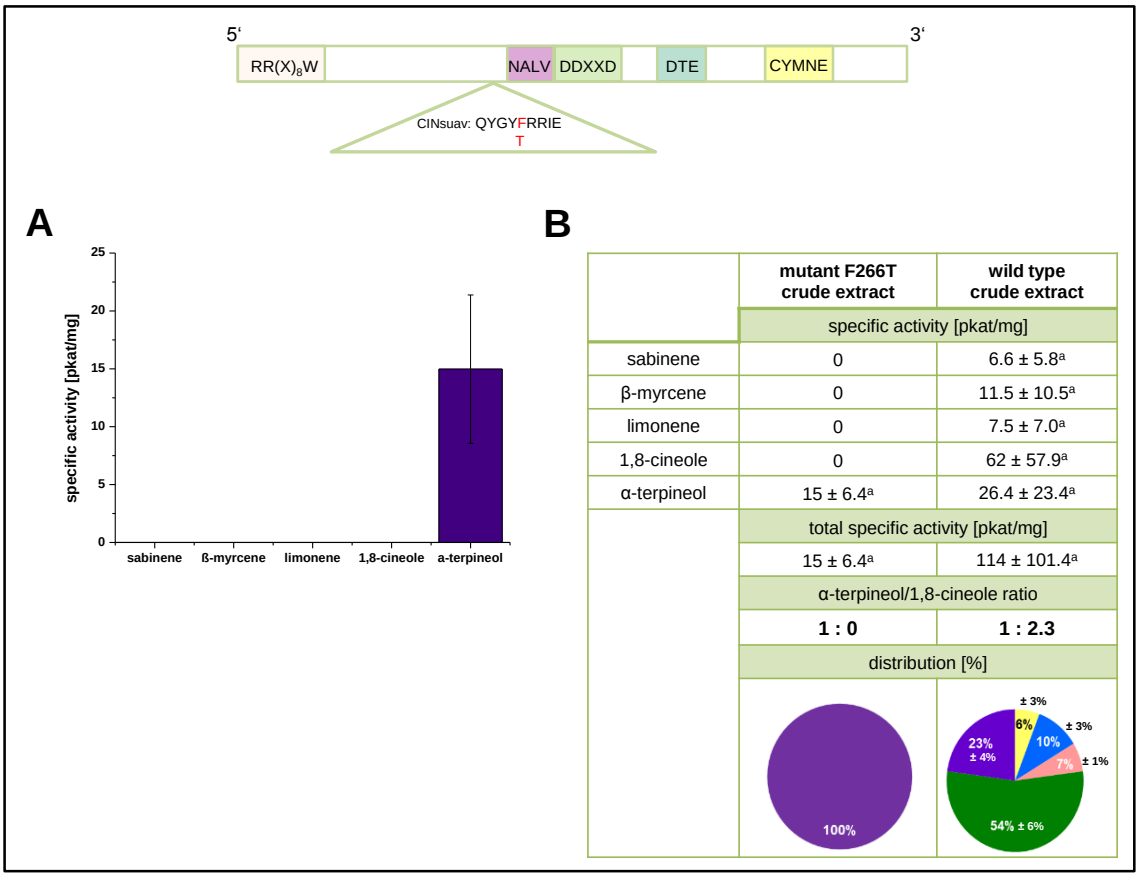
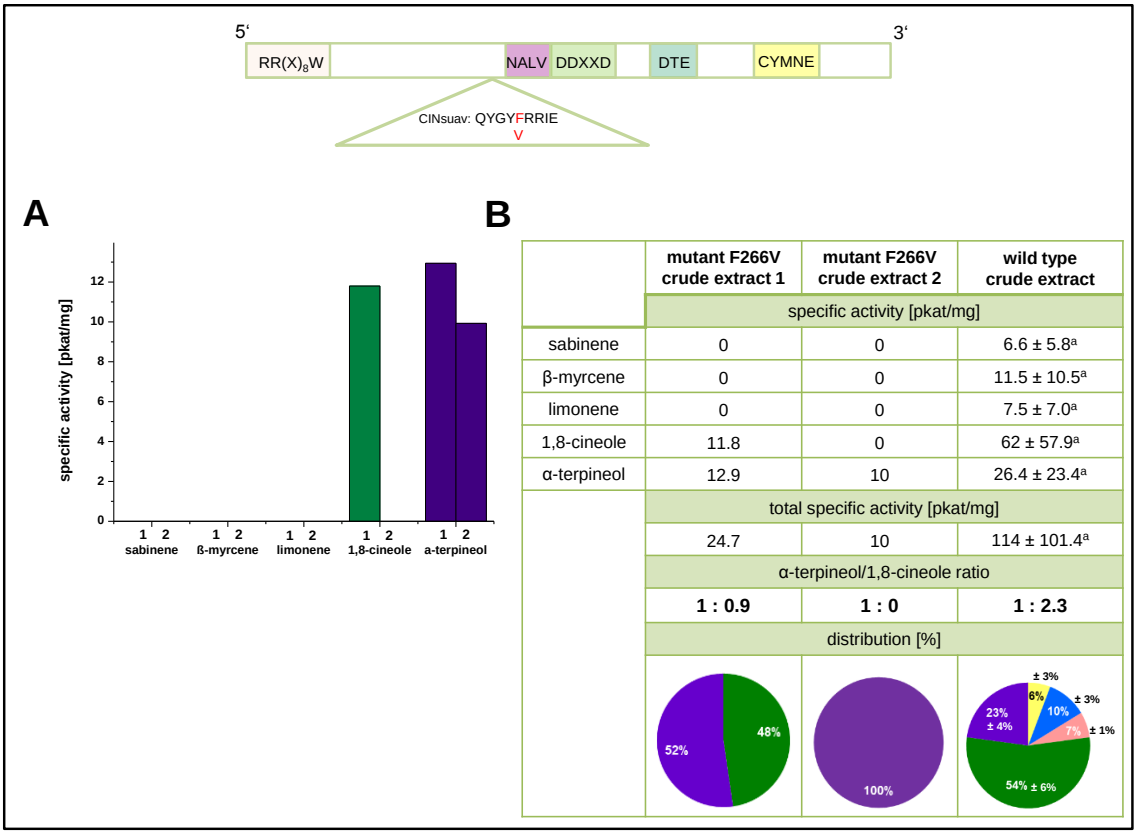


Figure 25: Analysis of the enzyme activity of the overexpressed mutant F266T of the 1,8-cineole synthase of *N. suaveolens* (n=3). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant F266T and the wild type enzyme. The error bars indicate the standard deviation. The values ± [a] indicate the standard deviation.

3.6.3 Mutant F266V

The mutation F266V is the substitution of a bulky and aromatic amino acid with a nonpolar, small, non-hydroxylated amino acid (Figure 26 A and B). This mutant was generated to examine the influence of hydrophobicity or hydrophilicity on the α-terpineol/1,8-cineole ratio. In the first experiment with the F266V mutant, two monoterpenoid compounds were detected, with specific activities of 12 pkat/mg for 1,8-cineole and 13 pkat/mg for α-terpineol. Consequently, the α-terpineol/1,8-cineole ratio was 1:0.9. In the second experiment, α-terpineol was detected, and the ratio of α-terpineol/1,8-cineole was 1:0. The total specific activity of the mutant enzyme was 10 pkat/mg and 24 pkat/mg in the first and second experiments, respectively, and was lower

than that of the wild type enzyme. Both experiments with the mutant F266V supported the observation that the reduced synthesis of 1,8-cineole (Figure 26, B) led to an increase in the synthesis of α-terpineol.



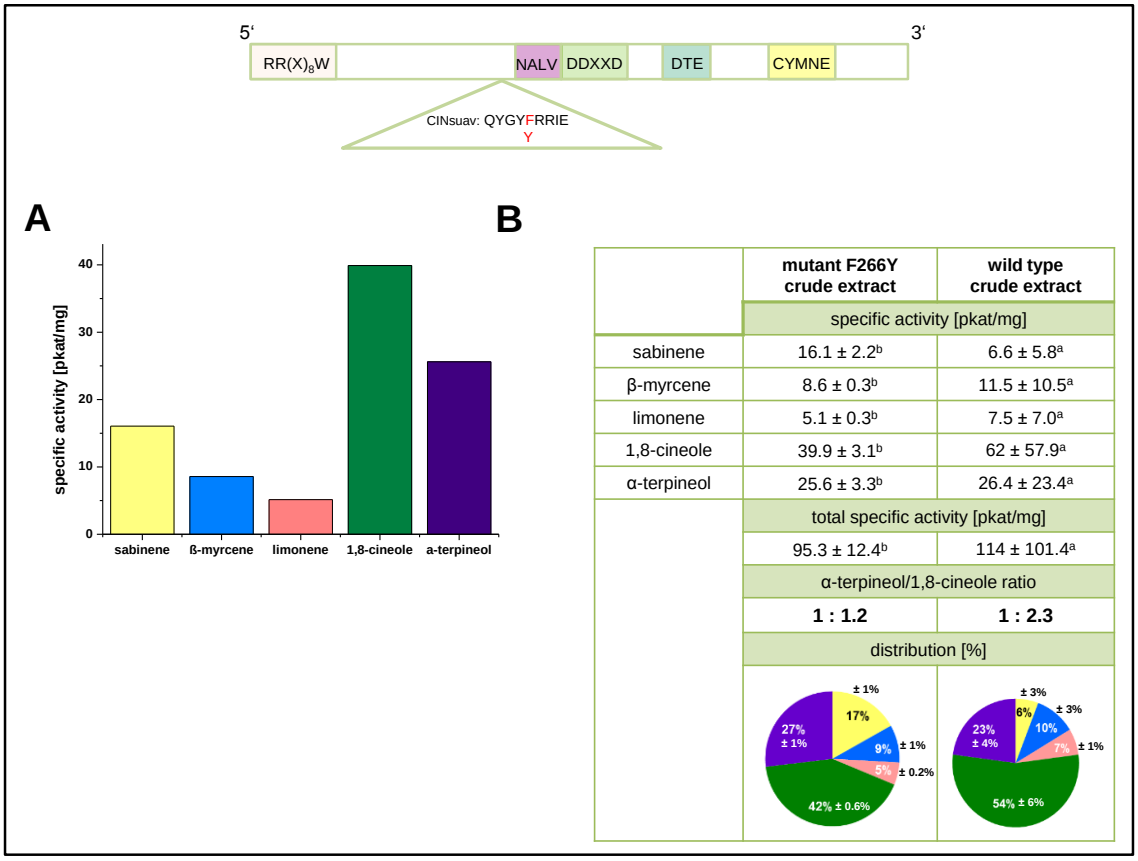
	mutant F266V crude extract 1	mutant F266V crude extract 2	wild type crude extract
	specific activity [pkat/mg]		
sabinene	0	0	6.6 ± 5.8[a]
β-myrcene	0	0	11.5 ± 10.5[a]
limonene	0	0	7.5 ± 7.0[a]
1,8-cineole	11.8	0	62 ± 57.9[a]
α-terpineol	12.9	10	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	24.7	10	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 0.9	**1 : 0**	**1 : 2.3**
	distribution [%]		

Figure 26: Analysis of the enzyme activity of the overexpressed mutant F266V of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant F266V and the wild type. The values ± [a] indicate the standard deviation.

3.6.4 Mutant F266Y

GC-MS analysis of the hexane extract of the mutant F266Y revealed the five monoterpenoid compounds described for the wild type enzyme (Figure 27 A and B; supplement 4). The specific activities were 39.9 pkat/mg for 1,8-cineole, 25.6 pkat/mg for α-terpineol, 16.1 pkat/mg for sabinene, 8.6 pkat/mg for β-myrcene and 5.1 pkat/mg for limonene. The specific activity for 1,8-cineole was reduced nearly twofold, while the specific activity for sabinene increased by a factor of two.

The mutant F266Y exhibited reduced synthesis of 1,8-cineole, as evident in the α-terpineol/1,8-cineole ratio of 1:1.6, and the total specific activity decreased slightly to 95.3 pkat/mg.



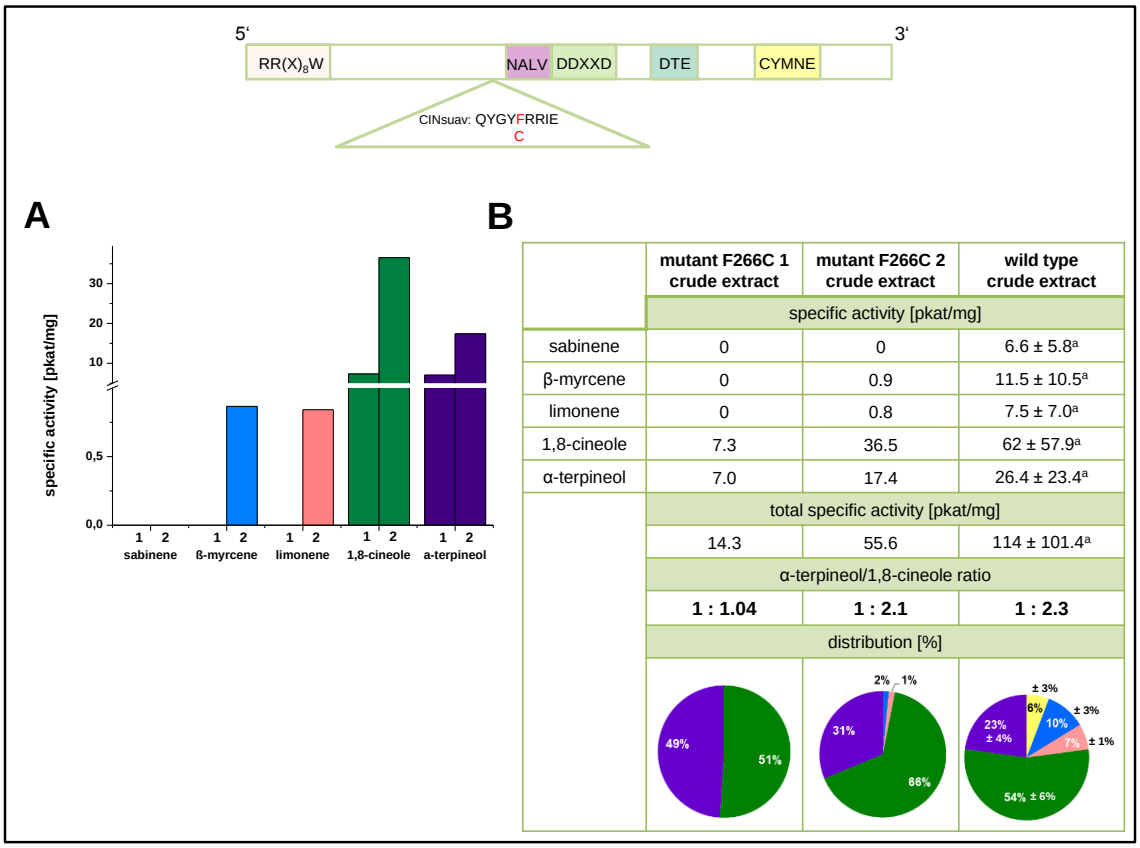
	mutant F266Y crude extract	wild type crude extract
	specific activity [pkat/mg]	
sabinene	16.1 ± 2.2[b]	6.6 ± 5.8[a]
β-myrcene	8.6 ± 0.3[b]	11.5 ± 10.5[a]
limonene	5.1 ± 0.3[b]	7.5 ± 7.0[a]
1,8-cineole	39.9 ± 3.1[b]	62 ± 57.9[a]
α-terpineol	25.6 ± 3.3[b]	26.4 ± 23.4[a]
	total specific activity [pkat/mg]	
	95.3 ± 12.4[b]	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio	
	1 : 1.2	**1 : 2.3**
	distribution [%]	

Figure 27: Analysis of the enzyme activity of the overexpressed mutant F266Y of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant F266Y and the wild type. The values ± [a] indicate the standard deviation and ± [b] indicate the mean deviation.

3.6.5 Mutant F266C

The phenylalanine at position 266 was substituted with cysteine, a small, polar, sulfhydryl-bearing amino acid, to test the hypothesis that the structure of this amino acid is decisive for more efficient synthesis of 1,8-cineole. The first analysis of the mutant enzyme F266C (Figure 28 A and B) indicated that the specific activities for 1,8-cineole (7,3 pkat/mg) and α-terpineol (7 pkat/mg) were nearly identical to one another but remarkably reduced compared to the wild

type enzyme. The α-terpineol/1,8-cineole ratio was balanced and reached 1:1.04. The specific activities in the second experiment were higher than those obtained in the first experiment but lower than those of the wild type enzyme. The total specific activities were reduced compared to that of the wild type enzyme, to 14.3 pkat/mg and 55.6 pkat/mg in the first and second experiment, respectively. The distribution of volatiles differed from that of the wild type enzyme: 66 % 1,8-cineole, 31 % α-terpineol, 2 % β-myrcene and 1 % limonene. However, the α-terpineol/1,8-cineole ratio, 1:2.1, was nearly identical to that of the wild type enzyme.



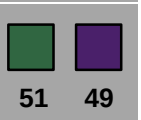
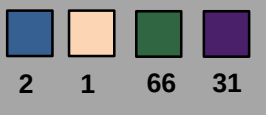
	mutant F266C 1 crude extract	mutant F266C 2 crude extract	wild type crude extract
	specific activity [pkat/mg]		
sabinene	0	0	6.6 ± 5.8[a]
β-myrcene	0	0.9	11.5 ± 10.5[a]
limonene	0	0.8	7.5 ± 7.0[a]
1,8-cineole	7.3	36.5	62 ± 57.9[a]
α-terpineol	7.0	17.4	26.4 ± 23.4[a]
	total specific activity [pkat/mg]		
	14.3	55.6	114 ± 101.4[a]
	α-terpineol/1,8-cineole ratio		
	1 : 1.04	**1 : 2.1**	**1 : 2.3**
	distribution [%]		

Figure 28: Analysis of the enzyme activity of the overexpressed mutant F266C of the 1,8-cineole synthase of *N. suaveolens* (n=2). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the enzyme in the crude extract. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the crude extract protein of the mutant F266C and the wild type. The values ± [a] indicate the standard deviation.

The analyses of the crude extracts of the mutant enzymes F266S, F266T, F266V, F266Y and F266C are summarized in Table 3. The mutant enzymes F266Y and F266C exhibited reduced synthesis of 1,8-cineole compared to the wild type enzyme, as indicated by α-terpineol/1,8-cineole ratios of 1:1.6 and 1:2.1, respectively. In one experiment with the mutant F266C, the α-terpineol/1,8-cineole ratio was balanced, at 1:1.04. Notably, analysis of the crude extracts of the mutants F266S, F266T and F266V indicated α-terpineol/1,8-cineole ratios of 1:0.5, 1:0 and 1:0.9, respectively, in contrast to the ratio of 1:2.3 for the wild type enzyme. For the mutated enzymes F266S, F266T and F266V, the monoterpenoid α-terpineol was synthesized as the major compound. The synthesis of 1,8-cineole was tremendously reduced or disturbed because the mutant F266T synthesized only α-terpineol. In the second experiment with the mutant F266V, α-terpineol was the only product. To confirm these results, the mutant enzymes F266S, F266T and F266V were further purified.

Table 3: Summary of the results for the five analyzed mutated enzymes at position 266 in the crude extract. The abbreviation of the mutation, the α-terpineol / 1,8-cineole ratios of the first and second experiments of the mutated enzymes, the total specific activity and the volatile distribution numbers below colored squares are listed and compared to the wild type crude extract. Color code, yellow: sabinene, blue: β-myrcene, wheat: limonene, green: 1,8-cineole and purple: α-terpineol.

crude extract	α-terpineol/ 1,8-cineole ratio (wild type enzyme crude extract: 1:2.3)	total specific activity [pkat/mg] (wild type enzyme crude extract: 114 ± 101.4 pkat/mg)	distribution [%] wild type enzyme crude extract: 6 10 7 54 23
F266S 1st	1:0.5	30.1 ± 10.1	13 7 12 22 46
2nd	1:0	13.4 ± 11.1	100
F266T	1:0	15.0 ± 6.4	100
F266V 1st	1:0.9	24.7	48 52
2nd	1:0	10.0	100

F266Y	1:1.2	95.3 ± 12.4	
F266C 1st	1:1.04	14.3	
2nd	1:2.1	55.6	

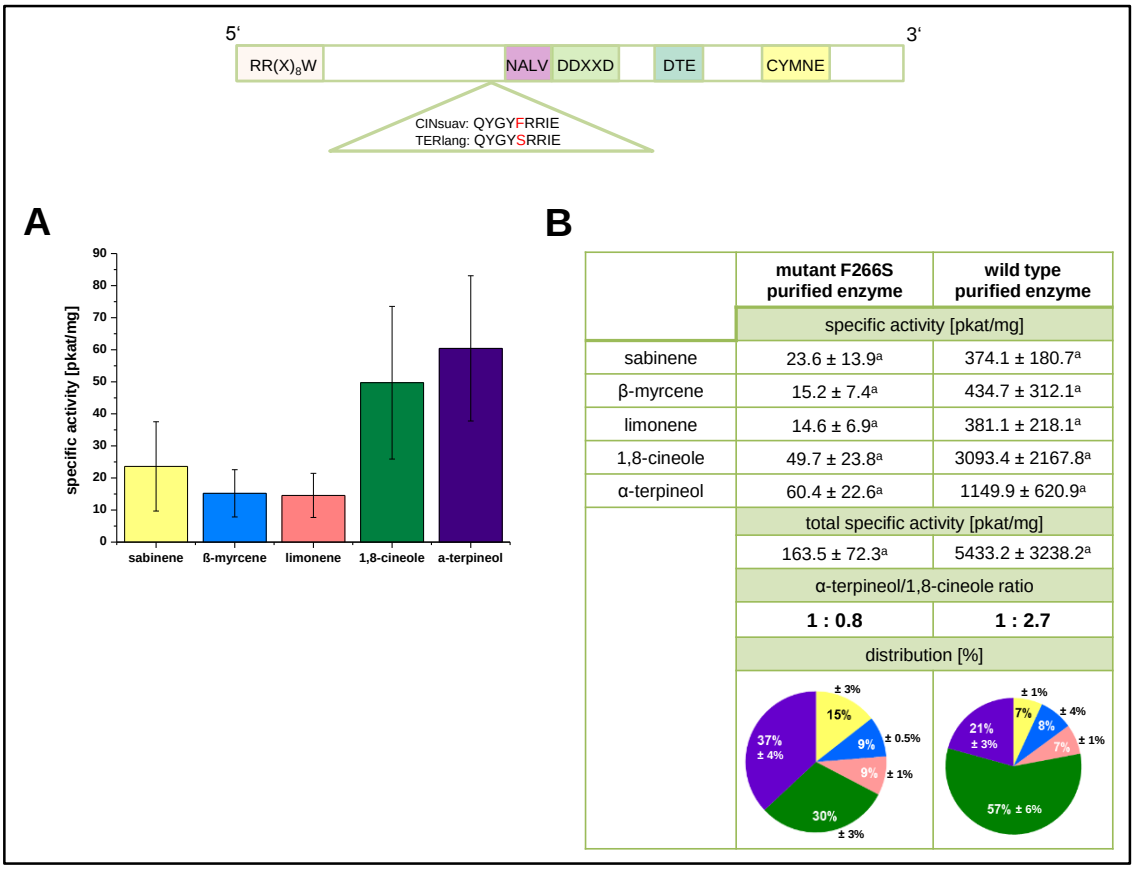
3.7 Analyses of the purified F266S, F266T and F266V enzymes

Despite repeated efforts, the purification of the enzymes F266S, F266T and F266V from the crude extracts presented in Figures 24, 25 and 26 was not successful, most likely due to the small quantity of active enzyme in the purified fractions. Therefore, the three mutants (F266S, F266T and F266V) were produced again, and the enzymes were purified and characterized (K_M, v_{max}, k_{cat}/K_M). GC-MS analysis of the crude extracts of the purified mutant enzymes F266S, F266T and F266V did not reveal any monoterpenoid compounds. However, based on the results of the previous experiments, the crude extracts of the regenerated mutants F266S, F266T and F266V were purified.

3.7.1 Purified mutant F266S enzyme

GC-MS analysis of the purified mutant enzyme F266S (Figure 29 A and B) confirmed some of the results measured for the enzyme in the crude extract. All five monoterpenoid compounds were detected, and α-terpineol, not 1,8-cineole, was the major compound. The total specific activity of the purified mutant enzyme F266S was dramatically decreased (163.5 pkat/mg) compared to the wild type enzyme (Figure 29 B). The specific activity was 60 pkat/mg for α-terpineol, 49.7 pkat/mg for 1,8-cineole, 23.6 pkat/mg for sabinene, 15.2 pkat/mg for β-myrcene and 14.6 pkat/mg for limonene. The distribution (%) was different compared to the wild type enzyme, and a switch of the α-terpineol/1,8-cineole ratio from 1:2.7 to 1:0.8 was observed. The results for the purified mutant

F266S demonstrated that synthesis of 1,8-cineole was decreased significantly compared to the wild type purified enzyme.



	mutant F266S purified enzyme	wild type purified enzyme
	specific activity [pkat/mg]	
sabinene	23.6 ± 13.9[a]	374.1 ± 180.7[a]
β-myrcene	15.2 ± 7.4[a]	434.7 ± 312.1[a]
limonene	14.6 ± 6.9[a]	381.1 ± 218.1[a]
1,8-cineole	49.7 ± 23.8[a]	3093.4 ± 2167.8[a]
α-terpineol	60.4 ± 22.6[a]	1149.9 ± 620.9[a]
	total specific activity [pkat/mg]	
	163.5 ± 72.3[a]	5433.2 ± 3238.2[a]
	α-terpineol/1,8-cineole ratio	
	1 : 0.8	**1 : 2.7**
	distribution [%]	

Figure 29: Analysis of the enzyme activity of the overexpressed purified mutant enzyme F266S of the 1,8-cineole synthase of *N. suaveolens* (n_{biol}=3). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the purified enzyme. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the purified enzyme of the mutant F266S and the wild type. The error bars and the values ± [a] indicate the standard deviation.

3.7.2 Purified mutant F266T enzyme

GC-MS analysis of the purified mutant enzyme F266T (supplement 4 and Figure 30 A and B) revealed three monoterpenoid compounds: sabinene, 1,8-cineole and α-terpineol. The specific activities of the purified mutant F266T were very low compared to the wild type enzyme: 85.4 pkat/mg for 1,8-cineole, 72.8 pkat/mg for α-terpineol and 24.3 pkat/mg for sabinene (Figure 30, A and B). The results obtained for the purified mutant enzyme F266T were inconsistent with those obtained for the enzyme in the crude extract (Figure 25), although in both cases three replicates were performed. Furthermore, the α-

terpineol/1,8-cineole ratios were not the same for the purified enzyme and the enzyme in the crude extract. However, the α-terpineol/1,8-cineole ratio of the purified mutant F266T, 1:1.2, was reduced compared to the wild type enzyme, indicating a decrease in the efficiency of the synthesis of 1,8-cineole (Figure 30 B) and reduced conversion of α-terpineol to 1,8-cineole.

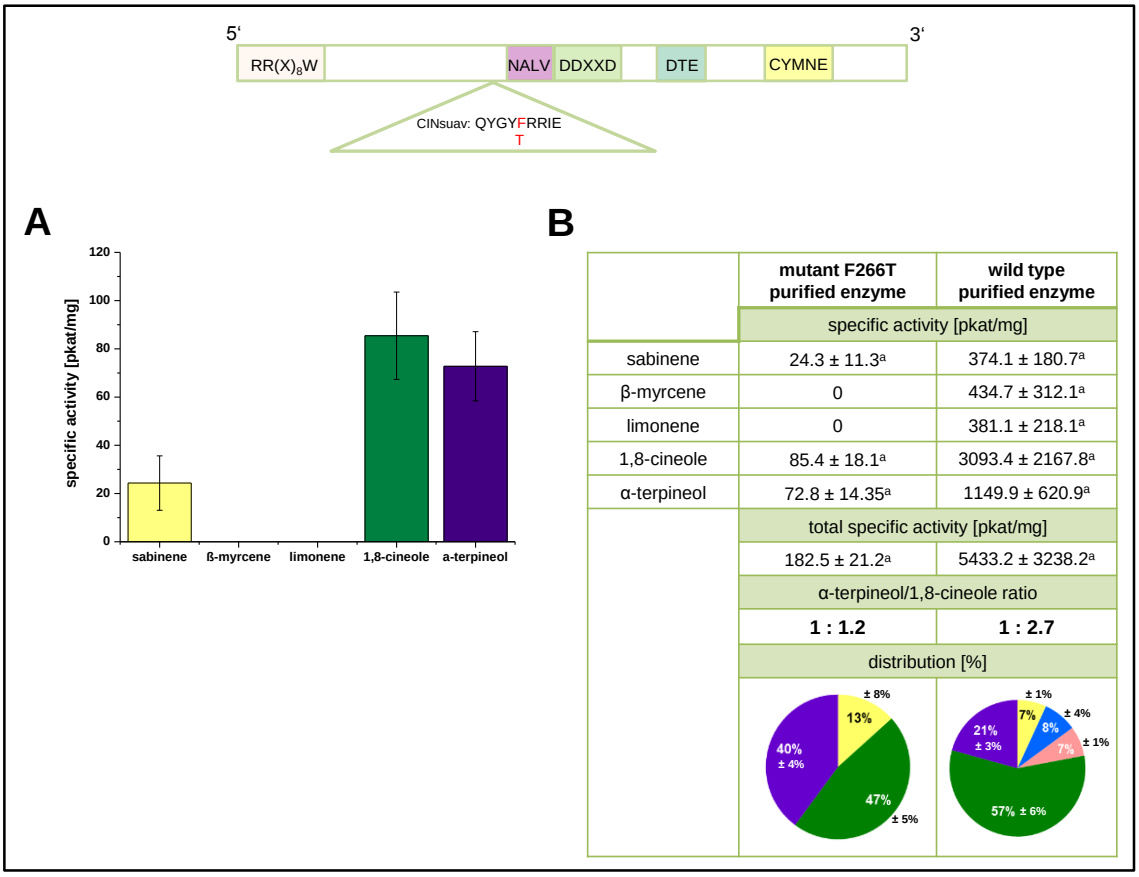


Figure 30: Analysis of the enzyme activity of the overexpressed purified mutant enzyme F266T of the 1,8-cineole synthase of *N. suaveolens* (n_{biol}=3). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the purified enzyme. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the purified enzyme of the mutant F266T and the wild type enzyme. The error bars and the values ± [a] indicate the standard deviation.

3.7.3 Purified mutant F266V enzyme

The results of the analyses of the purified mutant enzyme F266V (Figure 31 A and B) were quite similar to those obtained for the purified mutated enzyme F266T. GC-MS analysis of the purified mutant enzyme F266V (for chromatogram, see supplement 4) revealed all five monoterpenoid compounds, whereas only two compounds were detected for the enzyme in the crude extract

(Figure 26). The specific activities were very low compared to the wild type enzyme: 17.9 pkat/mg for sabinene, 10.9 pkat/mg for β-myrcene, 6.1 pkat/mg for limonene, 35.7 pkat/mg for 1,8-cineole, and 28.9 pkat/mg for α-terpineol. The α-terpineol/1,8-cineole ratio was 1:1.2, indicating that the efficiency of the cyclization reaction to 1,8-cineole was decreased compared to the wild type enzyme (1:2.7).

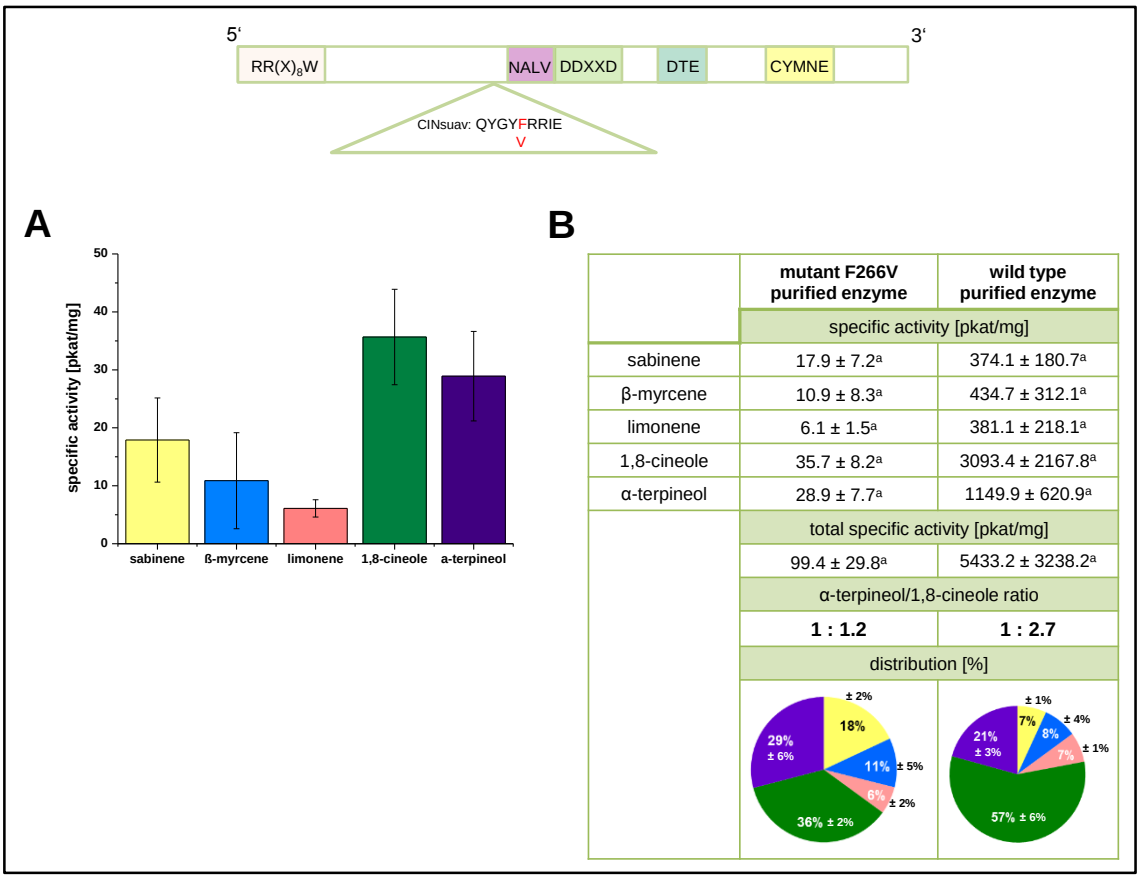


Figure 31: Analysis of the enzyme activity of the overexpressed purified mutant enzyme F266V of the 1,8-cineole synthase of *N. suaveolens* (n=3). Illustration ahead shows the position where the mutation was introduced. **A**: Specific activities in pkat/mg of the purified enzyme. The hexane phase of the enzyme assay with 10 µg enzyme was analyzed by GC-MS. **B**: Comparison of the specific activities, the α-terpineol / 1,8-cineole ratios and the distribution (%) between the purified enzyme of the mutant F266V and the wild type. The error bars and the values ± [a] indicate the standard deviation.

Table 4 presents a summary of the results for the amino acid alterations at position 266. Position 266 in the 1,8-cineole synthase of *N. suaveolens* appears to be crucial for the cyclization reaction to 1,8-cineole. The first analyses of the crude extracts of the mutants F266T and F266V revealed a switch in the α-terpineol/1,8-cineole ratio, whereas the analysis of the purified enzymes indicated tremendously reduced synthesis of 1,8-cineole, with a decrease of the

ratio to 1:1.2 compared to the wild type purified enzyme (1:2.7). The substitution with serine at position 266 leads to an inefficient cyclization reaction of 1,8-cineol, as indicated by an α-terpineol/1,8-cineole ratio of 1:0.8 for the purified mutant enzyme and consistent with the results of the mutant enzyme in the crude extract (Figure 24). In addition, the mutant enzyme F266T synthesized only sabinene, 1,8-cineole and α-terpineol, whereas the mutant enzymes F266V and F266S synthesized all five monoterpenoids observed for the wild type enzyme. The volatile distribution of the mutants F266T and F266V revealed that the proportion of α-terpineol increased to 29-40 % compared to the wild type purified enzyme. However, the total specific activities of the mutants F266S, F266T and F266V were decreased by a factor of 30-54 compared to the wild type purified enzyme.

Table 4: Summary of the results of the three analyzed mutations at position 266 in the purified fraction. The abbreviation of the mutation, the α-terpineol/1,8-cineole of the mutated enzymes, the total specific activity and the volatile distribution numbers below colored squares are listed and compared to the wild type purified enzyme. Color code, yellow: sabinene, blue: β-myrcene, wheat: limonene, green: 1,8-cineole and purple: α-terpineol. ± indicate the standard deviation.

purified enzyme	α-terpineol/ 1,8-cineole ratio (wild type purified enzyme: 1:2.7)	total specific activity [pkat/mg] (wild type purified enzyme: 5433 ± 3238.2 pkat/mg)	distribution [%] wild type purified enzyme: 7 8 7 57 21
F266S	1:0.8	163.5 ± 72.3	15 9 9 30 37
F266T	1:1.2	182.5 ± 21.2	13 47 40
F266V	1:1.2	99.4 ± 29.8	18 11 6 36 29

The results for the substitutions at position 266 indicate that the amino acids phenylalanine, tyrosine, cysteine, threonine and valine facilitate the reaction to the bicyclic monoterpenoid 1,8-cineole (Figure 32). By contrast, the mutant enzyme F266S promotes the synthesis of α-terpineol, even though position 266 is distant from the DDXXD motif (Figure 32).

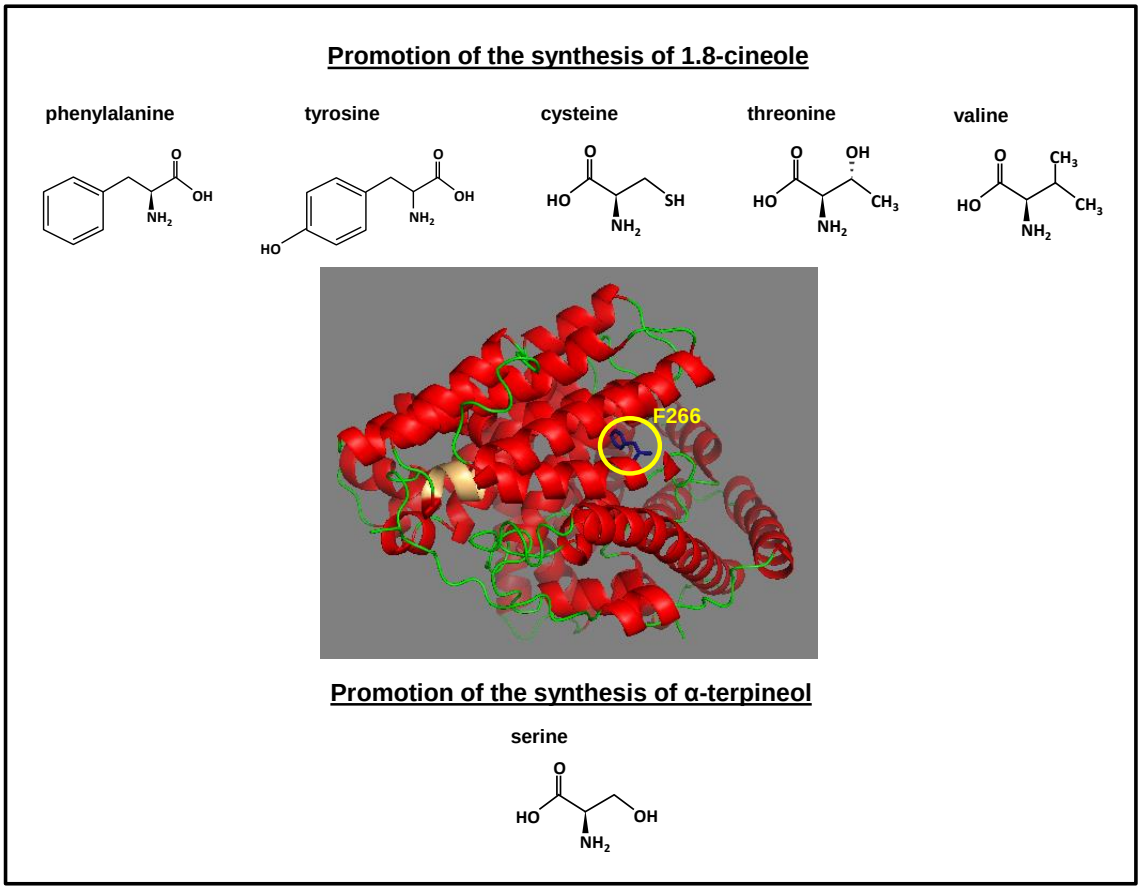


Figure 32: Location of position 266 in the protein model of the 1,8-cineole synthase from *N. suaveolens*. The protein model is shown as a helix-loop-helix illustration (red: helix and green: loop). The DDXXD motif is highlighted in light orange, and the phenylalanine (F) at position 266 is highlighted in blue and encircled in yellow.

3.7.4 Biochemical characterization of the purified wild type enzyme and the mutant enzymes F266S, F266T and F266V

The biochemical parameters of the overexpressed and purified wild type and mutant (F266S, F266T and F266V) enzymes were analyzed. The three mutant enzymes were characterized because their α-terpineol/1,8-cineole ratios were significantly different than that of the wild type enzyme. SDS-PAGE and

Western Blots with a specific antibody against the polyhistidine residue were performed to detect the overexpressed enzyme (Figure 33). The wild type and the mutant enzymes were approximately 68 kDa because the mutations involved only nucleotide substitutions or deletions of a maximum of two amino acids. Three elution fractions (1-3) after purification are shown. The strongest signal with a molecular mass of ~68 kDa was observed in fraction two.

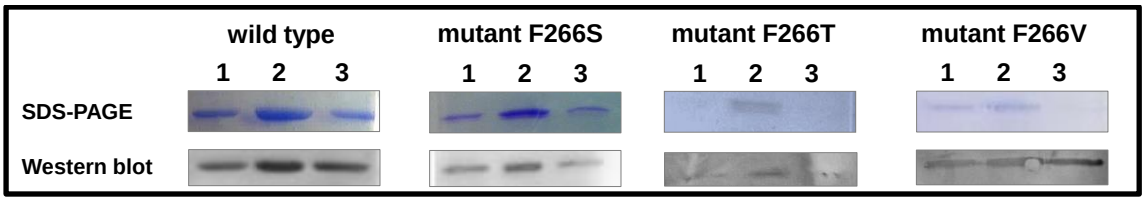


Figure 33: SDS-PAGE and Western blot analysis of the purified wild type and mutant (F266S, F266T, F266V) enzymes. The numbers above are the number of the eluted fraction. In the first row are presented the analysis of the SDS-PAGE stained with Coomassie Brilliant Blue R-250. Shown are the bands of the right size by ~68 kDa. The second row shows the PVDF membrane for the verification by Western Blot of the terpene synthase in the wild type, the mutant F266S, F266T and F266V. The used antibodies are the Anti-poly histidine as the primary antibody and the Anti-mouse antibody as the second antibody. A define volume (18 µl from 500 µl) from each fraction was applied on a 12,5 % gel. The complete SDS-PAGE and Western Blot analysis of the wild type enzyme and the mutant enzymes are in the supplement 5.

The Michaelis-Menten constant (K_M), maximum velocity (v_{max}), turnover number (k_{cat}), catalytic efficiencies and concentration of the active center of the enzyme (E_0) were determined for each of the purified enzymes (Table 5). The K_M values of the wild type and mutant F266S enzymes were in similar: 0.19 µM and 0.12 µM, respectively. The mutant enzymes F266V and F266T had much lower K_M values of 0.04 µM. The maximum velocity of the wild type enzyme and mutant enzymes were in the same range: 0.0012-0.008 µM/min. The turnover number indicates the number of substrate molecules that can be converted by an enzyme per second. The mutant enzyme F266S and the wild type enzyme converted 2.9-4.0 x 10^{-4} s^{-1} molecules of GPP per second to monoterpenoid products, whereas the mutants F266T and F266V were one order of magnitude less efficient (5.8 x 10^{-5} s^{-1} to 8 x 10^{-5} s^{-1}, respectively). The catalytic efficiency is the most meaningful parameter for characterizing enzymes. The catalytic efficiencies of the wild type enzyme and the mutant enzymes were in the same range, 1450-2416 s^{-1} x M^{-1}, indicating that the mutations at position 266 were not deleterious to enzymatic activity.

Table 5: Biochemical characterization of the wild type and three mutant enzymes F266S, F266T and F266V. The enzyme assay with radioactive ^{3}H-GPP was performed with 1 µg purified enzyme in a total volume of 50 µl (see material and methods). There results v_{max} [µM/min], E_0 [M], k_{cat} [s^{-1}], K_M [µM] and the catalytically efficiency k_{cat}/K_M [s^{-1} x M^{-1}] were calculated. To generate the data the Lineweaver-Burk plot was used (supplement 6). Number of the replicates/repeats were: wild type: n=1, mutant F266S: n_{biol}=3, mutant F266T: n_{biol}=3, mutant F266V: n=3.

	K_M [µM]	v_{max} [µM/min]	E_0 [M]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} x M^{-1}]
wild type	0.19	0.008	3.4×10^{-7} M	4.1×10^{-4}	2157
F266S	0.12	0.006		2.9×10^{-4}	2416
F266T	0.04	0.0012		5.8×10^{-5}	1450
F266V	0.04	0.0017		8×10^{-5}	2000

4. Discussion

Interesting results were obtained in the analysis of 19 mutants of the 1,8-cineole synthase of *N. suaveolens*. For some mutant enzymes (G7Q, C235G, M290L, H298D, I305T, N324D, A355S), the total specific activities were increased compared to the wild type enzyme, and all five monoterpenoids produced by the wild type enzyme were detected. Mutants that exhibited lower total specific activities than the wild type enzyme produced one, two, three or four monoterpenoids. For the mutant enzymes H298D, I305T and A355S, the α-terpineol/1,8-cineole ratio was approximately twofold higher (1:4, 1:3.6 and 1:3.4, respectively) than that of the wild type enzyme (1:2.3). These results indicate an enhancement of 1,8-cineole synthesis. For those mutant enzymes that synthesized all five monoterpenoids, the product distributions were the same as that of the wild type enzyme. The monoterpenoids α-terpineol and 1,8-cineole were always the major compounds, and sabinene, β-myrcene and limonene were the minor synthesized compounds.

The mutant F266S synthesized more α-terpineol than 1,8-cineole and displayed a switch in the α-terpineol/1,8-cineole ratio compared to the wild type enzyme. To further probe this result, four other mutants at position 266 were generated (F266T, F266V, F266Y and F266C). The analysis of the mutant enzymes in crude extracts revealed obviously decreased or converted α-terpineol/1,8-cineole ratios of 1:1.4, 1:1.2 and 1:0, respectively.

Surprisingly, the results obtained for the purified F266T and F266V mutant enzymes were inconsistent with the results obtained with the corresponding crude extracts. The analysis of the crude extracts of the mutant enzymes F266T and F266V also indicated a reversal of the α-terpineol/1,8-cineole ratio, whereas the purified mutant enzymes exhibited a ratio of 1:1.2. The total specific activities of the purified mutated enzymes were considerably lower than that of the wild type purified enzyme. However, the three purified mutant enzymes (F266S, F266T and F266V) exhibited approximately the same values of K_M, v_{max}, k_{cat} and k_{cat}/K_M as the purified wild type enzyme.

4.1 Protein dynamics cause conformational changes and altered enzyme activity and product outcome

As first established for ribonuclease A, the structure of an enzyme is determined by its amino acid sequence (Anfinsen, 1973). Alterations of the amino acid sequence influence the structure of an enzyme and therefore the flexibility of the protein. The results of this study should be considered in the context of dynamic nature of enzymes because proteins are not static but rather flexible (Bu and Callaway, 2011). As reviewed by Gerstein et al. (1994), X-ray crystal structures have demonstrated domain movements that reveal the high flexibility of proteins. Domain motions in proteins are essential for many protein functions. Particularly relevant to this study, domain motion influences catalysis and regulation of activity. Some domains in monoterpene synthases occupy conserved motifs which also have different functions (for details, see the introduction), e.g., the C-terminal DDXXD motif coordinates the binding of divalent metal ions and the N-terminal $RR(X)_8W$ motif is important for the isomerization of the substrate GPP. Many studies (Starks et al. 1997; Bohlmann, J., Meyer-Gauen, G., Croteau 1998; Christianson 2006; Kampranis et al. 2007) have demonstrated that mutations of specific motifs influence protein flexibility, product outcome and activity. These changes occur because mutations in the amino acid sequence affect the structure of the enzyme and subsequent conformational configurations triggered by protein dynamics. Protein dynamics and conformational changes are closely linked and are subsequently responsible for alterations in enzyme activity and product outcomes.

4.1.1 Amino acid substitutions influence enzyme activity and α-terpineol/1,8-cineole ratio

In one experiment, GC-MS analyses of the mutants G7Q, C235G, M290L, H298D, I305T, N324D and A355S indicated very high specific activities compared to the wild type enzyme. Histidine is a polar, positive charged amino acid, whereas aspartate is negatively charged. These amino acids are not typically localized inside proteins, but charge may be important for the active

site cavity. The local partial charge at the active site cavity may stabilize the carbocationic intermediates and therefore influence the catalytic mechanism (Starks et al. 1997; Lesburg 1997). The folding of the enzyme and the conformational configuration of the amino acids in the mutants may be different than those of the wild type enzyme, and substrate binding may proceed more efficiently when an aspartate is present at position 298 in the 1,8-cineole synthase of *N. suaveolens*. Liu et al. (2014) determined that, in *ent*-kaurene synthase from the bacterium *Bradyrhizobium japonicum*, the substrate binds to an active pocket near a cluster of aspartate and arginine residues and that these amino acids are essential for catalysis. The aspartate at position 298 in the 1,8-cineole synthase of *N. suaveolens* may influence the aspartate-rich DDXXD motif, which binds and coordinates divalent metal ions as co-factors, to allow the enzyme to catalyze the reaction more efficiently. López-Gallego et al. (2010) demonstrated for sesquiterpene synthases Cop4 and Cop6 from *Coprinus cinereus* that a disruption of the hydrogen bond between an asparagine and aspartate compromises catalytic efficiency of the mutants Cop4 and Cop6. They further suggest that this interaction is critical in positioning side chains for the optimal coordination of divalent metal ions. The aspartate at position 298 of the 1,8-cineole synthase of *N. suaveolens* may form a hydrogen bond with a proximally located asparagine (e.g., to an asparagine at position 314) and promote the catalytic efficiency and increase the specific activity due to the optimal coordination of the divalent metal ions.

The single base mutation M290L also resulted in higher specific activity compared to the wild type enzyme. Koshland (1958) postulated that enzyme and substrate are dynamic systems and interact with one another. The leucine substitution may alter the folding of the enzyme active site such that the substrate GPP fits better in this conformation of the active pocket, consistent with an induced-fit mechanism. Leucine is important for hydrophobic interactions inside the structure of the protein. The leucine at position 290 contains two methyl groups in its side chain, whereas methionine has a long side chain with a sulfur-containing group. The amino acids leucine and methionine are hydrophobic but the hydrophobic effect of both amino acids is different. The hydrophobicity of leucine is higher as described for methionine

(Matthews 2001). The hydrophobic effect avoids contact with water molecules and is described as the driving force in stabilization of proteins (Matthews 2001). ‘Large-to-small’ substitutions of hydrophobic amino acids (leucine to alanine mutations) in T4 lysozyme induced large conformational changes inside the core. A ‘large-to-small’ replacement can change the protein structure surrounding the replacement and as a result a cavity is formed (Matthews 2001). This cavity cause the loss of stability of the protein (Eriksson et al. 1992; Xu et al. 1998; Matthews 2001). The mutation M290L in the 1,8-cineole synthase of *N. suaveolens* may not result in a cavity and promote the protein stability and consequently increase the activity of the mutant enzyme compared to the wild type enzyme.

The activity of the double mutant M290L/H298D was completely unexpected; whereas the single mutants M290L and H298D exhibited high enzyme activities, the double mutant exhibited decreased activity. Both mutations are near the DDXXD motif (Figure 10 B) and are part of the putative catalytically active pocket. The introduced leucine and aspartate residues may influence the structure of the enzyme such that substrate binding was altered and catalytic activity was reduced. For an aspartate aminotransferase from *E. coli*, Almo & Smith (1994) demonstrated that mutation of R298 to aspartate resulted in low catalytic efficiency and decreased substrate binding affinity. Although aspartate aminotransferase belongs to a different class of enzymes than the 1,8-cineole synthase of *N. suaveolens*, enzymes are dynamic systems in general. These dynamic motions influence enzyme catalysis by affecting substrate binding affinity and product release. Protein dynamics should be recognized as an important contributor to enzyme catalysis (Karplus 1984; Hammes 2002; Pervushin et al. 2007). Conformational changes influence the dynamics of enzymes and, consequently, their activity. In addition, some domains of a protein influence protein dynamics more than others. The C-terminal domain is important for the activity (Peters & Croteau 2003; Christianson 2006). The mutant enzyme EE335/336-deleted in which the glutamates at position 335/336 were eliminated exhibited decreased total specific activity compared to the wild type enzyme. Negatively charged amino acids may be required at positions 335/336, which are located in the C-terminal domain of the 1,8-cineole synthase

of *N. suaveolens*, for catalytic activity. Köllner et al. (2004) and Yoshikuni et al. (2006) also concluded for sesquiterpene synthases that a disturbance of function due to amino acid substitutions reduced the activity of proteins and altered the product spectra.

The replacement of a nonpolar amino acid with a polar amino acid (I305T and A355S) could have also a great impact on the catalytically active site due to the reactive hydroxyl group of threonine. Bartelt (2014) used semi-empirical calculations to demonstrate that the hydroxyl group of threonine at position 278 in the 1,8-cineole synthase from *N. forgetiana* stabilizes via the formation of a hydrogen bond the nucleophilic attack of a water molecule at the C7 α-terpinyl. The threonine at position 305 may serve a stabilizing role that allows the reaction cascade to 1,8-cineole proceed with increased specific activity.
In terpene synthases, 'special' amino acid residues function as plasticity residues. Plasticity residues occur in and around the active site of terpene synthases and are responsible for catalytic elasticity (Starks et al. 1997; Greenhagen et al. 2006; Li et al. 2013). Alterations of plasticity residues can change the product specificity (Yoshikuni et al. 2006; Xu et al. 2007; Kampranis et al. 2007). Li et al. (2013) demonstrated that substitution of plasticity residues can improve the catalytic efficiencies a sesquiterpene synthase. The threonine at position 399 in the sesquiterpene synthase from *Artemisia annua* is important for catalytic specificity. However, substitution with serine at position 399 increased the yield of amorpha-4,11diene. Alanine 355 may function as a plasticity residue in the active pocket of the 1,8-cineole synthase of *N. suaveolens*, and its mutation to serine improved the catalytic efficiency with increased synthesis of monoterpenoids.
The mutant N324D represents the alteration of a polar amino acid to an acidic amino acid. Klodt (2013) observed an increase in the specific activity of a similar mutant (Q220E) of the α-terpineol synthase from *N. langsdorfii* compared to the wild type α-terpineol synthase. Zilske (2012) discussed in her thesis that acidic amino acids can bind metal ions. With respect to the aspartate-rich motif, the binding of divalent metal ions (Mg^{2+} or Mn^{2+}) increased the enzymatic activity. This increase may be due to an enhancement of metal ion binding by the DDXXD motif due to the N324D mutation. Further, Zilske

(2012) proposed that a negatively charged amino acid residue interacts with positively charged amino acid residues to form ionic bonds that stabilize the structure of the enzyme. The carbocationic intermediates may also be stabilized, which would allow catalysis to proceed at an increased rate.

The mutation G7Q is located at the N-terminus in a variable position of the $RR(X)_8W$ motif. This residue is far away from the putative active pocket of the 1,8-cineole synthase of *N. suaveolens* (Figure 10 B). Amino acids surrounding the active pocket can greatly affect enzyme catalysis (Back & Chappell 1996; Hyatt & Croteau 2005, Greenhagen et al. 2006). Starks et al. (1997) determined that the surrounding layers of the tobacco 5-epi-aristolochene synthase and the vetispiradiene synthase from *Hyoscyamus muticus* determine the product specificity due to a distinct conformation of the active site. The formation of hydrogen bonds between glutamine and water molecules or intramolecular interactions with other polar groups may stabilize globular proteins (Myers & Pace 1996), resulting in improved substrate conversion.

The mutants G7Q, C235G, M290L, H298D, I305T, N324D and A355S of the 1,8-cineole synthase of *N. suaveolens* exhibited increased total specific activity compared to the wild type enzyme. Despite this increased specific activity, these mutations have not evolved in the sequence of the wild type enzyme of *N. suaveolens*, possibly because monoterpenoid production requires substantial energy. High synthesis of monoterpenoids may not be advantageous, as evidenced by the lack of evolution of these residues in the amino acid sequence. Anfinsen & Scheraga (1975) concluded that an amino acid sequence evolves to achieve a particular biological advantage so that favorable intermolecular interactions can decrease the free energy of the system, forming folded aggregates. This conclusion is in accordance with Pare & Tumlinson (1999), who proposed sensitive synthesis and safe storage of volatiles because increased synthesis could be costly and possibly toxic to the plant.

Second measurements and analyses of the mutant enzymes T279A, M290L/H298D, H298D, I305T, N324D, EE335/336-deleted and A355S revealed very low total specific activities compared to the wild type enzyme. Only α-terpineol and 1,8-cineole were detected. The specific activities of these enzymes may have been low that the amount of synthesized compound did not

reach the detection limit of the GC-MS system, preventing the determination of all five monoterpenoids. Roeder et al. (2007) have previously discussed the detection limit of GC-MS for β-pinene for the 1,8-cineole synthase of *N. suaveolens*. Brosemann (2011) reported variations between experiments in the product profile of overexpressed 1,8-cineole synthase from *N. forgetiana*. In some measurements, she detected the expected five monoterpenoids of the so-called “cineole cassette” (Raguso et al. 2006), while only β-linalool or only two monoterpenoids were detected in other experiments. She suggested that the overexpression conditions might also be responsible for the different product spectra, which merits careful consideration. The mutation could influence the product outcome, but the conditions of the enzyme assay may also influence the mutant enzyme. Optimal conditions are only known for the wild type 1,8-cineole synthase of *N. suaveolens*.

4.1.2 Sabinene and β-myrcene are absent in the product profiles of some mutant enzymes

In the product profiles of the mutant enzymes A355S, N324D, T279A and M290L/H298D, sabinene was not detected, but the other four compounds (β-myrcene, limonene, 1,8-cineole, α-terpineol) were detected. This result may reflect the detection limit of the GC-MS system or a disturbance of the synthesis of sabinene. The formation of the thujyl-cation (the cationic intermediate of sabinene) involves a hydride shift of the α-terpinyl cation via the terpinen-4-yl cation (see introduction, Figure 5). It is possible that the mutation T279A leads to a sufficient stabilization of the α-terpinyl cation and the rearrangement to terpinen-4-yl cation is not necessary to reach a more stable carbocation and this could prevent the synthesis to sabinene.

The disturbed synthesis of the specific carbocationic intermediates of monoterpenoid compounds may also be responsible for the absence of β-myrcene in the spectrum of the mutant enzyme Y446A. This mutation may have affected the synthesis of acyclic products, possibly by disturbing the deprotonation of the geranyl cation to form β-myrcene (see introduction, Figure 5) (Dudareva et al. 2003; Chen et al. 2003). By contrast, the reaction cascade

to form the α-terpinyl cation proceeds as usual, as evidenced by the production of the four cyclic monoterpenoids (sabinene, limonene, 1,8-cineole and α-terpineol). The hydroxyl group of tyrosine at position 446 may be involved in the protonation of the α-terpinyl cation because substitution with the methyl group of alanine led to a decrease in the specific activity of the mutant enzyme. In the 5-*epi*-aristolochene synthase from tobacco, elimination of the tyrosinyl hydroxyl group at position 520 (which is important for the protonation of the intermediate germacrene A) led to a 10-fold decrease in k_{cat} compared to the wild type enzyme (Rising et al. 2000). They proposed a decrease in the rate of product release from the enzyme. β-myrcene may also be derived from the linalyl cation (Degenhardt et al. 2009), and its absence may be due to a failure of deprotonation. This mutation may influence the synthesis of acyclic monoterpenoids but did not affect the synthesized quantity of cyclic products.

4.1.3 Involvement of F300 and Y502 in the catalytically active pocket

In the mutant enzymes F300I and Y502V, aromatic amino acids were converted to hydrophobic amino acids without a benzene ring. These mutants were unable to synthesize monoterpenoids, which suggests that these amino acids play a role in the catalytically active pocket. These mutations may influence the structure of the enzyme in a manner that hinders substrate binding, resulting in a blockage of the reaction. Brandt et al. (2009) demonstrated that aromatic amino acid residues in the active site play an important role in recognition, stabilization and orientation of the α-terpinyl cation intermediate in prenyl cyclases. The α-terpinyl cation is the precursor of all cyclic monoterpenoids. The missing benzene ring of the aromatic amino acids (Phe or Tyr) may lead to a destabilization of this cation and, consequently, alteration of the volatile profile (Brandt et al. 2009, Bartelt 2014). This scenario may apply for the mutant F300I. For the mutant Y502V, other effects may be relevant, such as disturbance of the protonation of the carbonyl double bond of the monoterpenoid α-terpineol such that the reaction to 1,8-cineole failed. In this case, the reaction cascade was terminated after formation of the α-terpinyl cation. Bartelt (2014) determined that a tyrosine at the equivalent position 496 in the 1,8-cineole

synthase from *N. forgetiana* is responsible for protonation of the carbonyl double bond of α-terpineol. Bartelt (2014) proposed protonation via a charge-relay-system from the hydroxyl group of the α-terpineol via the hydroxyl group of tyrosine 496. Little & Croteau (2002) demonstrated that a substitution of tyrosine at position 566 with phenylalanine in the γ-humulene synthase from grand fir (*Abies grandis*) modified the product spectra, with reduced proportions of sibirene and β-gurjunene. In the 3D protein model (Figure 10), Y502V is in the near of the DDXXD motif, consistent with its hypothesized involvement in the catalytically active pocket.

4.1.4 Multi-product formation

Monoterpene synthases can synthesize a broad range of products from a single substrate. Different carbocationic intermediates are formed during catalysis, and the favored product depends on the termination mechanism (Christianson 2008; Degenhardt et al. 2009; Chen et al. 2014). The dynamic of proteins enable active pocket flexibility, which is critical for monoterpene synthesis. Dynamic transitions of the enzyme may be correlated with the amino acid composition in and around the active site, which may be unique for each synthesized monoterpenoid. A specific amino acid composition influences the protein dynamic and favors the synthesis of sabinene, limonene, 1,8-cineole and other monoterpenes. All reaction mechanisms (e.g., deprotonation, hydride shift, water capture) for the synthesis of multiple monoterpenes could be favored by the enzyme. Different reaction pathways could be favored by one enzyme molecule or many enzyme molecules. Many enzyme molecules, not a single enzyme molecule, are present inside a plant as well as in the *in vitro* enzyme assay. The different enzyme molecules may favor different reaction mechanisms. Kampranis et al. (2007) observed that the combination of the contour and dynamics of the active site, which facilitate the different isomerization steps, is more important than the precise positioning of a reactive group.

Analysis of the product outcomes of the mutants of the 1,8-cineole synthase of *N. suaveolens* (e.g., G7Q, C235G, M290L) revealed that the mutations did not

influence the distribution of the synthesized monoterpenoids, which was often identical to that of wild type. The synthesized by-products (sabinene, β-myrcene and limonene) were always present in lesser amounts than 1,8-cineole and α-terpineol. The activity of the mutant enzymes during synthesis changed, but not the ratio between the minor products and major products (1,8-cineole and α-terpineol). The mutant enzymes G7Q, C235G, and M290L also primarily synthesized 1,8-cineole and α-terpineol, as described for the wild type enzyme. In general, terpene synthases synthesize multiple minor products in addition to the major products (Chen et al. 2014). Chen et al. (2014) proposed that the minor product activities of multi-product enzymes provide the raw material for the evolution of novel enzymes because the minor activities can become dominant activities (Chen et al. 2014). The authors further elucidated for the genus *Oryza* that the diversification of the synthesized terpenoids was important for the adaptation of plants to specialized niches and was principally driven by positive Darwinian selection.

Christianson (2008) explained this product promiscuity of terpenoid synthase via the diverse carbocationic reaction mechanism and the permissiveness of the active site.

The synthesis of more 1,8-cineole or α-terpineol in multi-product enzymes could also reflect the different stereoselectivity of the intermediates. The wild type 1,8-cineole synthase of *N. suaveolens* synthesizes more 1,8-cineole than α-terpineol, but the wild type α-terpineol synthase of *N. langsdorfii* and the mutant F266S of the 1,8-cineole synthase of *N. suaveolens* synthesize more α-terpineol than 1,8-cineole. Krause et al. (2013) compared two multi-product monoterpene synthases from the sabinene hydrate chemotype in *Thymus vulgaris*, TPS6 and TPS7. Both convert the α-terpinyl cation via the terpinene-4-yl cation and the sabinyl cation into sabinene hydrate. TPS6 forms monoterpenes of the *S*-configuration and TPS7 forms monoterpenes of the *R*-configuration. The amino acid identity between TPS6 and TPS7 was 85 %, and the amino acid Asn-350 in TPS6 corresponds to Ile-346 in TPS7. Site-directed mutagenesis, overexpression and incubation with GPP as the substrate revealed that the TPS6 mutant Asn350Ile altered its stereospecificity to that of TPS7. They concluded that the initial conformation of GPP determines the opposite stereochemistry of TPS6 and TPS7. The conversion of GPP to LPP is

stereospecific and influenced by the helical fold of the native substrate (Krause et al. 2013). In addition, Croteau et al. (1986) determined that right-hand folded GPP facilitates the formation of (3*S*)-LPP and left-hand folded GPP promotes the formation of (3*R*)-LPP. The serine at position 266 in the 1,8-cineole synthase of *N. suaveolens* may influence the conformation of the active pocket. The mutant enzyme F266S converts GPP to the α-terpinyl cation and into a favorable configuration for the synthesis of more α-terpineol. Therefore, the configuration of the α-terpinyl cation that is preferred in the α-terpineol synthase from *N. langsdorfii* and the 1,8-cineole synthase from *N. suaveolens* should be determined. For the 1,8-cineole synthase from *S. officinalis*, 1,8-cineole is formed via the R-configuration of the α-terpinyl cation (Croteau et al.1994). The 1,8-cineole synthase of *N. suaveolens* and the α-terpineol synthase of *N. langsdorfii* may favor different stereospecific intermediates and thus influence major and minor product formation.

4.2 Position 266 plays a crucial role in the cyclization reaction to 1,8-cineole

4.2.1 The mutation F266S promote the synthesis of α-terpineol

A single amino acid substitution, deletion or insertion could alter the enzyme structure and, consequently, enzymatic activity and product release. Garms et al. (2012) demonstrated that a single amino acid substitution near the active pocket altered the product outcome for the sesquiterpene synthases SbTPS1 and SbTPS2 from *Sorghum bicolor*. The single base mutation F266S is located on the same helix as the DDXXD motif, where the active pocket of monoterpene synthases is putatively located, but on the opposite side (see Figure 32). The amino acid replacement to a hydroxylated, polar amino acid changed the product outcome dramatically, with a shift in the α-terpineol/1,8-cineole ratio to 1:0.8. α-Terpineol was the main compound in the multi-product release of the mutant enzyme F266S and was synthesized with an average specific activity of 60.4 pkat/mg, whereas the specific activity for 1,8-cineole was only 49.7 pkat/mg. Position 266 in combination with the amino acid substitution had a great impact on the enzyme activity and specificity. Klodt (2013)

demonstrated that the product profile of the reverse mutant S264F of the α-terpineol synthase from *N. langsdorfii* also changed. 1,8-Cineole was detected as the major compound, whereas the wild type α-terpineol synthase synthesized significantly more α-terpineol. The α-terpineol/1,8-cineole ratio of the purified enzyme S264F was balanced (1:1.01), whereas the crude extract exhibited a distinct converted ratio of 1:1.8. The analysis of the crude extract and purified enzyme of the mutant S264F of the α-terpineol synthase from *N. langsdorfii* revealed different product profiles; sabinene and limonene were not detected for the purified enzyme S264F, in contrast to the results obtained for the mutant F266S of the 1,8-cineole synthase from *N. suaveolens*. The mutation to serine at position 266 influenced the shift in the major monoterpenoid compound but not the whole product spectrum. The by-products sabinene, limonene and β-myrcene were detected for the purified mutant enzyme F266S, although, the specific activities of the purified mutant enzyme F266S were 20- to 70-fold lower than those of the purified wild type enzyme (Figure 29 and 11). These results suggest that the shift away from 1,8-cineole production may be due to an actual loss of function of the enzyme. It is unclear if the mutant enzyme F266S influences the catalytic mechanism either through direct alteration of the active cavity or by influencing amino acids in the second sphere of the active site. Second-sphere amino acids can affect the active site geometry and catalysis of an enzyme (Hyatt & Croteau 2005; Greenhagen et al. 2006). Lodeiro et al. (2004) reported that mutation of a second-sphere amino acid in oxidosqualene cyclase was critical for the catalytic distinction between cycloartenol synthase and lanosterol synthase activity. The authors noted that mutations outside the active site influenced the hydrogen-bonding network and, consequently, altered the product profile. This explanation could also apply for the altered product profile of the mutant F266S. However, Bartelt (2014) demonstrated in a hypothetical protein model of the 1,8-cineole synthase from *N. forgetiana* that phenylalanine 266 is more than 10 Å away from the α-terpinyl cation and therefore argued that a direct influence on the production of 1,8-cineole may not occur. In an RNase A enzyme, Watt et al. (2007) demonstrated that mutation of histidine 48, which is located 18 Å from the active site, to alanine resulted in a loss of protein motion in an isotope-sensitive region of the enzyme. Watt et al. (2007) further demonstrated that this histidine influenced

protein motion, and mutation to alanine limited the conversion of substrate to product. Bartelt (2014) assumed that phenylalanine 266 of the 1,8-cineole synthase of *N. forgetiana* influenced the active site indirectly by altering the plasticity of the enzyme via replacement of an aromatic, hydrophobic amino acid (phenylalanine) with a small, hydrophilic, hydroxylated amino acid (serine). This substitution might manipulate surrounding helices that extend to the active site and therefore affect catalysis (Bartelt 2014). This scenario may also occur in the 1,8-cineole synthase of *N. suaveolens* if the phenylalanine at position 266 was substituted to serine; the introduction of a hydroxyl group might result in increased synthesis of α-terpineol. Wu et al. (2006) demonstrated that substitution of the phenylalanine at position 445 of the oxidosqualene-lanosterol synthase from *Saccharomyces cerevisiae* with a polar side chain influenced the product spectra. Their analyses supported the Johnson model (Johnson 1991). Johnson (1991) hypothesized that cation-π interactions between carbocationic intermediates and an enzyme can be replaced by a polar or electrostatic side chain to alter the product spectrum.

The mutation F266S converted the 1,8-cineole synthase of *N. suaveolens* to a α-terpineol synthase because α-terpineol was synthesized as the major compound. Kampranis et al. (2007) attempted to convert a monoterpene synthase into a sesquiterpene synthase by mutating the important asparagine 338 to alanine to accommodate farnesyl pyrophosphate in the active site cavity for efficient sesquiterpene synthesis. The substitution F266S in the 1,8-cineole synthase of *N. suaveolens* is hypothesized to make the active pocket of the mutant enzyme larger. The new geometry optimized the minimal free energy of the mutant enzyme and facilitated substrate binding and subsequently nucleophilic attack of the water molecule on the α-terpinyl cation. This enhancement increased the optimal conversion to α-terpineol as the major compound in the product spectrum. Bartelt (2014) attempted to use semi-empirical calculations to study the production of α-terpineol and 1,8-cineole by the 1,8-cineole synthase of *N. forgetiana*. An optimized geometry shortened the distance between the C7 of the α-terpinyl cation and the oxygen of the water molecule, potentially leading to a surplus of energy. Mutational analysis of a triterpene synthase (cycloartenol synthase) revealed that an enlarged active cavity led to alternative folding, which facilitated premature deprotonation by

shifting the A-ring closure to an amino acid that participates in the reaction (Segura et al. 2003).

4.2.2 Analysis of additional mutations at position 266

The substitution of phenylalanine with serine at position 266 in the 1,8-cineole synthase of *N. suaveolens* led to the increased detection of α-terpineol. Further mutational analyses at position 266 were performed to determine if the properties or structures of amino acids were responsible for the change in the product profile. The substitution of phenylalanine with tyrosine (F266Y, Figure 27) did not result in a reversal in the product outcome compared to the wild type enzyme, as expected. Tyrosine is an aromatic amino acid like phenylalanine but has a hydroxyl group in the side chain. Interestingly, 1,8-cineole remained the major product in the F266Y mutant. Therefore, continuation of the reaction to 1,8-cineole may depend on the structure and not the polarity of the amino acid. Notably, the benzene ring at position 266 of the enzyme is relevant to the production of more 1,8-cineole. The catalytic mechanism of terpene synthases depends on the stabilization of the carbocationic intermediate (Lesburg 1997; Starks et al. 1997; Rynkiewicz et al. 2001; Brandt et al. 2009). The product specificity of terpene synthases is guided by the aromatic amino acid side chain, which stabilize cations via cation-π interactions (Brandt et al. 2009). In addition, Starks et al. (1997) and Lesburg et al. (1997) revealed that aromatic residues of the active site of terpene synthases (sesquiterpene synthases) participate in carbocation stabilization via cation-π interactions. The π-electrons of the benzene ring interact with and stabilize the carbocationic intermediates so that the reaction cascade to 1,8-cineole can proceed. The benzene ring has a permanent quadrupole moment (Figure 34). *Ab initio* calculations of the electrostatic potential surface of the benzene ring indicated a positive charge (colored in blue) at the periphery and a negative charge (colored in red) on the surface of the benzene ring (Dougherty 1996). Therefore, the α-terpinyl carbocation can interact with the negatively charged surface of the benzene ring of tyrosine to form non-covalent cation-π interactions.

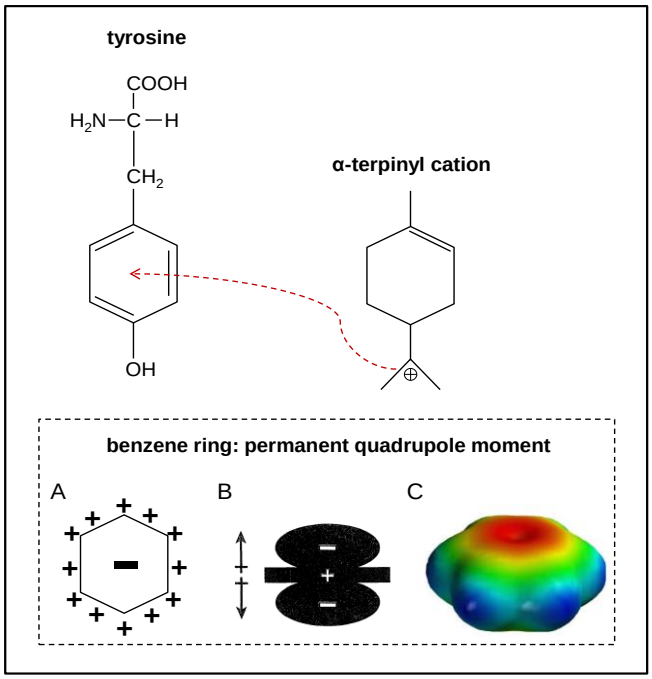


Figure 34: Cation-π interaction between the α-terpinyl cation and the benzene ring of tyrosine. The positive charge of the α-terpinyl cation interacts with the delocalized electrons on the surface of the benzene ring of tyrosine (red dotted arrow). The benzene ring has a permanent quadrupole moment. A and B show simple illustrations of the quadrupole moment with positive charge at the periphery and negative charge on the surface of the benzene ring. C illustrates the *ab initio* calculation of the electrostatic potential of the benzene ring surface. Red is negatively charged; blue is positively charged. (Dougherty 1996, modified).

Oliveira & Esteves (2011) demonstrated that the stabilization of carbocations reached a plateau when interaction with a benzene ring occurred. Cation-π interactions are noncovalent, weak electrostatic interactions and occur in many biological systems, e.g., acetylcholine receptors, K^+ channels and cyclase enzymes that synthesize steroids (Burley & Petsko 1986; Dougherty & Stauffer 1990; Mecozzi et al. 1996). Molecular docking analyses identified a cannabinoid receptor type 2 with a putative binding site for (*E*)-*β*-caryophyllene (Gertsch et al. 2008). These calculations indicated the presence of π-π stacking interactions between the C4-C5 double bonds of (*E*)-*β*-caryophyllene and the amino acids F117 and W258 (Gertsch et al. 2008). In addition, crystal structure analysis of the bornyl diphosphate synthase from *S. officinalis* revealed that the amino acids phenylalanine 578 and tryptophan 323 in the active site stabilize the positive charge at C8 of the (*R*)-α-terpinyl cation during catalysis (Whittington et al. 2002). The analysis of the mutant enzyme F266Y supports the hypothesis that the structure and not the properties of the amino acid at position 266 is decisive for the synthesis of more 1,8-cineole. To further elucidate the role of serine at position 266 in the synthesis of more α-terpineol, the mutants F266C, F266T and F266V were generated.

The comparison of the corresponding amino acid at position 266 in sequences of α-terpineol synthases from *Magnolia grandiflora* (Lee & Chappell 2008) or

Vitis vinifera (Martin & Bohlmann 2004) revealed the presence of a cysteine. The product profiles of the two α-terpineol synthases contained more α-terpineol, suggesting that cysteine at position 266 influences the product profile toward increased α-terpineol. However, the mutant enzyme F266C of *N. suaveolens* did not exhibit an altered product outcome compared to the wild type enzyme because cineole remained the major volatile. It was hypothesized that cysteine creates tight covalent bonds such that the reactive sulfhydryl group forms disulfide bridges that stabilize the intermediates. This stabilization is different than that described for the benzene ring and may promote the synthesis of 1,8-cineole. Interestingly, the *Citrus unshiu* 1,8-cineole synthase also harbors a cysteine at position 266 (Shimada et al. 2005). However, the α-terpineol synthases from *Magnolia grandiflora* and *Vitis vinifera* both contain a cysteine at the corresponding position but synthesize more α-terpineol. These observations confirm that the product pattern cannot be predicted by the amino acid sequence (Köllner et al. 2008; Frick et al. 2013).

The replacement of phenylalanine with threonine (a hydrophilic, hydroxylated amino acid) did not lead to a change in the product outcome. Threonine is larger than serine and contains a methyl group as well as a hydroxyl group. This substitution may not alter the hydrogen-bonding network in a positive manner for the synthesis of catalyze more α-terpineol. Liu et al. (1993) performed *ab initio* calculations and observed an on-face hydrogen bond between the hydroxyl side chain of threonine 13 and the aromatic ring of tyrosine 6 in the active site of the isoenzyme 3-3 of glutathione transferase. This type of hydrogen bond appear to affect catalysis by contributing a second-layer interaction that influences the interaction between the hydroxyl group of tyrosine 6 and the sulfur of bound glutathione anion (Liu et al. 1993). Semi-empirical measurements of the synthesis of 1,8-cineole by the 1,8-cineole synthase of *N. forgetiana* revealed that a tyrosine at position 496 protonates the double bond of α-terpineol (Bartelt 2014), followed by continuation of the reaction to 1,8-cineole. In the 1,8-cineole synthase of *N. suaveolens*, a threonine at position 266 might interact also with another tyrosine near the putative active site and trigger the protonation of the double bond of α-terpineol to continue the reaction to 1,8-cineole.

The analysis of the mutation to valine at position 266 further confirmed that amino acid structure and not hydrophobicity or hydrophilicity influences the synthesis of 1,8-cineole.

The biochemical characterization of the purified F266S, F266T, F266V and the wild type enzymes revealed typical values of K_M k_{cat} and v_{max} for terpene synthases. Faraldos et al. (2012) reported similar values for the (E)-*β*-farnesene synthase from *Mentha* x *piperita* (Table 5). For the substrate FDP (farnesyl diphosphate), a K_M of 1.8 µM and a turnover number of 0.028 s^{-1} were observed. Demissie et al. (2012) reported a turnover number of 8.8 x 10^{-3} s^{-1} and a K_M of 5.75 µM for the 1,8-cineole synthase isolated from *Lavandula* x *intermedia*. The measured K_M of the 1,8-cineole synthase from *A. thaliana* was 0.2 µM, with a turnover number of approximately 0.001 s^{-1} (Chen et al. 2004).
Interestingly, the catalytic quotients k_{cat}/K_M of the mutant enzymes F266S, F266T and F266V were all in the same range and as that of the wild type enzyme (Table 5), indicating that mutations at position 266 did not influence the efficiency of the enzyme. The values of k_{cat} and K_M for the F266T enzyme were obviously decreased compared to the wild type and mutant F266S enzymes (Table 5). However, Fersht (1977) concluded that an increased K_M and k_{cat} are expected in enzyme systems where k_{cat} is generally thought to be related to the rate of product release from the active site of the enzyme. A higher K_M explains the weaker binding of the substrate to the enzyme and results in a weaker binding of the products to the enzyme and a higher rapid rate of product release, which is expressed by a higher k_{cat}. The lower K_M and k_{cat} of the F266T mutant enzyme may indicate stronger binding of the products to the enzyme and a decreased rate of product release and explain the detection of only three and not five compounds in the product spectrum of the purified mutant enzyme F266T.

4.3 Putative reaction mechanism for 1,8-cineole

The mutant enzyme F266S was the only mutant that synthesized more α-terpineol than 1,8-cineole, resulting in a switch of the α-terpineol/1,8-cineole ratio (1:0.5 crude extract, 1:0.8 purified enzyme). Evaluating F266S in combination with the other substitutions at position 266 (tyrosine, cysteine, threonine, valine) enabled the formulation of a hypothetical reaction mechanism for the synthesis of 1,8-cineole, which is illustrated in Figure 35 A. The α-terpinyl cation plays a central role in the formation of cyclic monoterpenoid compounds (e.g., 1,8-cineole, α-terpineol). The C7 atom of the α-terpinyl cation undergoes nucleophilic attack by a water molecule to form α-terpineol. The redundant proton is proposed to transfer to a proton acceptor. The semi-empirical calculation for α-terpineol synthesis by the 1,8-cineole synthase of *N. forgetiana* indicated that a putative catalytic diad consisting of histidine 502 and glutamate 249 acts as the proton acceptor (Bartelt 2014). This scenario could also be hypothesized for the synthesis of α-terpineol by the 1,8-cineole synthase of *N. suaveolens*. The activation of α-terpineol is accompanied by protonation at the double bond of α-terpineol. This protonation is likely attributable to amino acids with proton donor qualities (e.g., tyrosine). Bartelt (2014) hypothesized for the 1,8-cineole synthase of *N. forgetiana* that the proton of α-terpineol is orientated toward the hydroxyl group of tyrosine 496. He further proposed a transfer of the proton to tyrosine 496, which in turn protonates the double bond of α-terpineol.
The resulting tertiary C1 carbocation eliminates a proton at the hydroxyl group, resulting in an intermediate with a partially negative charged oxygen atom (Figure 35 A). Nucleophilic attack of the negatively charged oxygen C8 atom on the cation of C1 results in the formation of the ether bond of 1,8-cineole. Proton loss and nucleophilic attack between C8 and C1 may proceed together (personal communication with R. Bartelt).
Based on the first results for the crude extracts of the enzymes containing mutations at position 266, it was assumed that the benzene ring was important because of carbocation stabilization via cation-π stacking. The benzene ring of phenylalanine (or the substituted tyrosine) at position 266 stabilizes the tertiary C1 carbocation intermediate via cation-π interactions, and the reaction

mechanism continues to 1,8-cineole. A serine at position 266 leads to the synthesis of more α-terpineol than 1,8-cineole. This substitution leads to a rearrangement of the amino acid composition in the active site such that the synthesis of α-terpineol would be preferred. The energetic conditions of the enzyme favor the increased synthesis of α-terpineol. Thus, the synthesis to 1,8-cineole is reduced because of the absence of the benzene ring of the phenylalanine that stabilizes the tertiary C1 carbocation.

The hypothesis that a small, hydroxylated amino acid (e.g., threonine) at position 266 is responsible for the synthesis of more α-terpineol than 1,8-cineole was disproved. The results obtained for the crude extracts of the mutant enzyme F266T revealed the production of more α-terpineol, but the purified enzyme synthesized more 1,8-cineole. The same result was observed for the mutant enzyme F266V. The analyses of the crude extract and purified enzyme of the mutant F266S were consistent and indicated more α-terpineol than 1,8-cineole in the product profile. These results demonstrate that only the specific amino acid substitution of serine at position 266 in the 1,8-cineole synthase of *N. suaveolens* can switch the α-terpineol/1,8-cineole ratio. The small side chain of serine containing a hydroxyl group seems to be essential for the synthesis of more α-terpineol because the additional methyl group in the side chain of threonine reduced the synthesis of more α-terpineol.

4.4 Synthesis of 1,8-cineole via the α-terpinyl hydronium ion (Wise et al. 2002) instead of α-terpineol

Several studies (Chen et al. 2004; Shimada et al. 2005; Kampranis et al. 2007; Degenhardt et al. 2009) have describe the synthesis of 1,8-cineole via the monoterpenoid α-terpineol. The putative pathway A (Figure 35 A) also requires the formation of α-terpineol, which would undergo a new activation so that the reaction continued to 1,8-cineole. The hypothesized pathway A (Figure 35 A) was first assumed when analyzing the results of the mutant enzymes at position 266 in the crude extract. However, the analysis of the purified enzymes did not confirm the assumption of pathway A, therefore a second pathway (Figure 35 B) described by Wise et al. (2002) was considered. The hypothesis of Wise et al.

(2002) suggests that the reaction to 1,8-cineole in *S. officinalis* proceeds via an α-terpinyl hydronium ion (Figure 35 B). The attack of a water molecule on the α-terpinyl cation results in an unstable intermediate that protonates itself at the double bond so that a fourth carbocation is formed. Proton release occurs at the end of the reaction and not during the synthesis to 1,8-cineole, as postulated for pathway A (Figure 35 A). In scenario B (Wise et al. 2002), the proton stabilizes the α-terpinyl hydronium ion, preventing the formation of α-terpineol. The probability of proton attack on the double bond of the α-tepinyl hydronium ion must be higher than that of release to form α-terpineol. The proton is transferred to an amino acid in the active site that acts as a proton acceptor, such as a double bond-containing amino acid (e.g., tyrosine, histidine, tryptophan).

Experiments with labeled water molecules have demonstrated that water is the only source of the oxygen atom in the ether bond of 1,8-cineole (Croteau et al. 1994). Labelled α-terpineol was not converted to 1,8-cineole, and the conversion of α-terpinyl pyrophosphate to 1,8-cineole failed. These results indicate that the synthesis to 1,8-cineole proceeds independent of α-terpineol in the 1,8-cineole synthase of *S. officinalis* (Croteau et al. 1994).

For the 1,8-cineole synthase from *A. thaliana*, Chen et al. (2004) suggested that most of the α-terpineol undergoes specific internal addition that may be facilitated by protonation of the double bond. This suggetion would support the synthesis of 1,8-cineole via α-terpineol (pathway A, Figure 35). However, can α-terpineol protonate the double bond itself? If this occurred, all of the α-terpineol would be converted and not detected in the product profile, which was not the case. The compound α-terpineol was always detected for the analyzed mutants. These results indicate that “self-protonation” of the α-terpineol by its own hydroxyl group is not possible.

The 1,8-cineole synthase from *Citrus unshiu* is a single-product monoterpene synthase that synthesizes 97.2 % 1,8-cineole (Shimada et al. 2005), further suggesting that the production of 1,8-cineole via α-terpineol does not occur. The lack of α-terpineol detected in the product spectrum supports the hypothesis that formation of 1,8-cineole does not proceed via α-terpineol.

The formation of other cyclic monoterpenoids (e.g., sabinene) does not proceed via a finished monoterpenoid structure but via carbocationic intermediates, supporting the hypothesis that the formation of 1,8-cineole is independent of α-

terpineol. Pinene is synthesized via the α-terpinyl cation and pinyl-cation (Croteau et al. 1989, Bohlmann et al. 2002). γ-Terpinene synthesis is based on the conversion of the α-terpinyl cation via 6,7-hydrid shift and deprotonation to the γ-terpinen-4-yl cation (LaFever & Croteau 1993, Degenhardt et al. 2009).

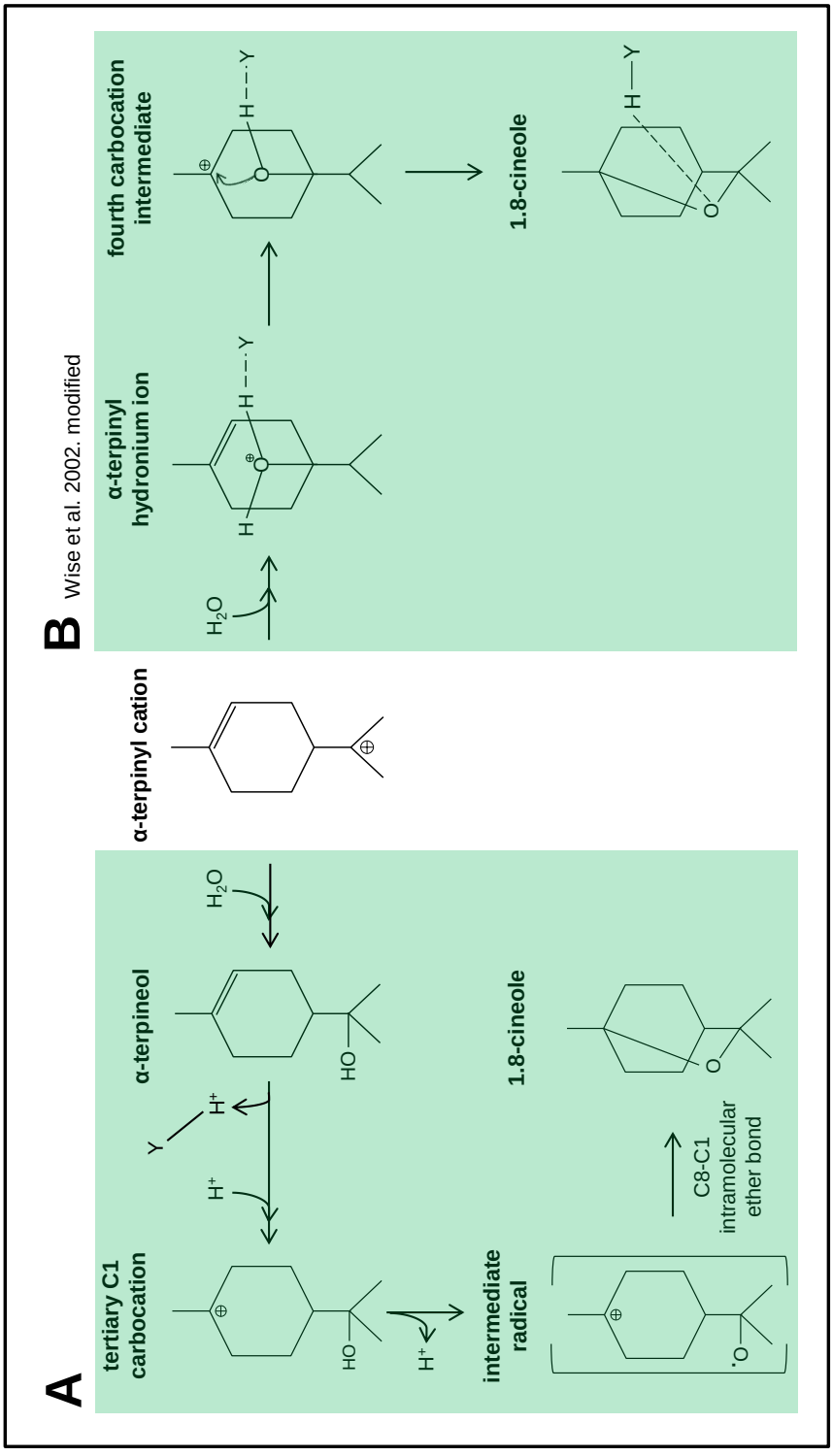


Figure 35: Putative cyclization reaction of the 1,8-cineole synthase from *N. suaveolens*. A: The α-terpinyl cation is hydrolyzed and converted to α-terpineol. The redundant proton could be transferred on a proton acceptor (Y). The following steps are hypothetical to form 1,8-cineole from α-terpineol. The double bond of the α-terpineol is protonated, and the tertiary C1-carbocation is formed. The C1-carbocation is subsequently deprotonated, nucleophilic attack from the negatively charged oxygen atom (compound in brackets) to the cation proceeds, and 1,8-cineole is formed. **B:** If a water molecule attacks at C7 of the α-terpinyl cation, the protons will not be released, and α-terpinyl hydronium ion is formed. Y indicates a proton acceptor in the active site. The broken lines indicate incomplete proton transfer. The protonation at the double bond of the α-terpinyl hydronium ion results in the formation of a fourth carbocation intermediate. The oxygen at C8 attacks the C1 cation of the fourth carbocation intermediate. The proton is transferred on an amino acid acceptor, and 1,8-cineole is formed

4.5 Synthesis of 1,8-cineole via α-terpineol (pathway A) versus synthesis of 1,8-cineole via α-terpinyl hydronium ion (pathway B)

Figure 35 suggests two putative pathways for the 1,8-cineole synthesis in *N. suaveolens*. Do these putative pathways A and B lead simultaneously to 1,8-cineole or is one of the pathways preferably or even solely responsible for the synthesis to 1,8-cineole? Based on the results regarding the mutations at position 266, pathway B could be proposed for the synthesis of 1,8-cineole. The replacement of phenylalanine by serine revealed a shift of the α-terpineol/1,8-cineole ratio towards α-terpineol when compared to the wild type enzyme. Nevertheless, the synthesis to 1,8-cineole did not cease completely but decreased considerably whereas the synthesis to α-terpineol increased. The mutation F266S obviously promoted the synthesis to α-terpineol via α-terpinyl cation and disturbed the synthesis to 1,8-cineole via the α-terpinyl hydronium ion. This conclusion is supported by the fact that the mutation F266Y did not change the product distribution compared to the wild type enzyme. The benzene ring of these two aromatic amino acids was assumed to stabilize the tertiary C1 carbocation of the activated α-terpineol by cation-π interactions when considering pathway A. However, this interaction was not necessary for 1,8-cineole production as shown by replacing phenylalanine by small aliphatic amino acids or polar amino acids (F266V, F266T, F266C). These mutant enzymes displayed also an unchanged product distribution compared to the wild type enzyme. 1,8-cineole was still the major compound although the cation-π stack was missing for the tertiary C1 carbocation stabilization which would be necessary for proceeding pathway A. Also α-terpineol was detected in the product profiles of the mutant enzymes F266C, F266T and F266V. Therefore I would propose that the synthesis of α-terpineol and 1,8-cineole proceeds independently from each other. Pathway A ends with the formation of α-terpineol. There is no carcocation formation to synthesize 1,8-cineole. 1,8-cineole is synthesized as shown in pathway B via an α-terpinyl hydronium ion. The question why the substituted serine did influence the product distribution still remains to be answered.

5. Summary

Aim of this study was the analysis of the cyclization reaction to 1,8-cineole of the 1,8-cineole synthase of *Nicotiana suaveolens*. α-terpineol has been considered so far as the precursor of the bicyclic compound 1,8-cineole. The α-terpineol synthase of *N. langsdorfii* synthesizes five monoterpenoids with α-terpineol as the major compound whereas the 1,8-cineole synthase of *N. suaveolens* synthesizes the same five monoterpenoids with 1,8-cineole as major compound. Therefore these multi-product enzymes, the 1,8-cineole synthase of *Nicotiana suaveolens* and the α-terpineol synthase of *N. langsdorfii*, were aligned. This comparison revealed 53 differences. It was hypothesized that one or more of these varying amino acids influence the product spectrum. Nineteen mutants of the 1,8-cineole synthase of *N. suaveolens* were generated by site-directed mutagenesis in order to substitute the amino acids of the 1,8-cineole synthase with amino acids of the equivalent position of the α-terpineol synthase of *N. langsdorfii*. The comparison of the gas chromatographic - mass spectrometric product profiles of the mutant enzymes with the wild type enzyme revealed insights about the impact of the substituted amino acid on the cyclization reaction to 1,8-cineole. Several mutations of the 1,8-cineole synthase of *N. suaveolens* reveal higher (e.g., M290L, C235G, G7Q) or lower (e.g., Y446A, T279A, EE335/336-deleted) specific activities or influence the product outcome (e.g., M290L/H298D) compared to the wild type enzyme. However the α-terpineol/1,8-cineole ratio was not inverted compared to the wild type enzyme. Only the mutant enzyme F266S carrying a phenylalanine replacement by serine showed an inverted product spectrum compared to the wild type enzyme. α-terpineol was the major compound in the product profile of the mutant F266S. The generation of four other mutants (F266C, F266V, F266T, F266Y) at the position 266 showed that the existence of an outside hydroxyl group influences the cyclization reaction to 1,8-cineole. It is hypothesized that the synthesis of 1,8-cineole proceeds independent of α-terpineol and via α-terpinyl hydronium ion (Wise et al. 2002).

6. Outlook

The aim of this study was the analysis of the cyclization reaction to 1,8-cineole of the 1,8-cineole synthase of *Nicotiana suaveolens*. The generated and analyzed mutant enzyme F266S revealed an increased share of α-terpineol in the product spectrum instead of 1,8-cineole compared to the wild type enzyme. The generation of four other mutants (F266C, F266V, F266T, F266Y) at this position 266 showed that the existence of a terminal hydroxyl group of serine changed the α-terpineol / 1,8-cineole ratio and influence the synthesis to 1,8-cineole compared to the wild type enzyme. However, what is so special about serine? In general it is difficult to analyze a putative mechanism of product formation using site-directed mutagenesis. Especially, the exchange of a single amino acid often reveals unexpected and barely allegeable results. Additional experiments using the cineole synthase of *N. forgetiana* revealed that the mutation T278A also led to an increased α-terpineol synthesis compared to the wild type cineole synthase of *N. forgetiana* (Hippauf, personal communication, March 2015). Bartelt (2014) hypothesized that the substitution of threonine 278 would disturb the nucleophilic attack of the water molecule to the α-terpinyl cation. Since this reaction is assumed to be the precondition for α-terpineol formation (figure 35). It could be expected that this mutation would hinder α-terpineol production. However, the mutant enzyme T278A showed an increased α-terpineol synthesis (Hippauf, personal communication, March 2015). This shows that site-directed mutagenesis could give a hint about a putative role of certain amino acids in reaction mechanisms of product formation but the analysis of the crystal structure of the wild type cineole synthase as well as of the mutated enzymes are essential for final conclusions. The crystal structure would give more insight in the reaction mechanism. Molecular docking experiments with the substrate GPP in the structure of the enzyme could show which amino acid positions influences the activity of the enzyme and which amino acids might actually form in the active pocket. Up to now, there are only three monoterpene synthases crystallized: the multi-product bornyl diphosphate synthase from *S. officinalis* (Whittington et al. 2002), the single product limonene synthase from *Mentha spicata* (Hyatt et al. 2007) and the multi-product 1,8-cineole synthase from *S. fruticosa* (Kampranis et al. 2007). To

understand the cyclization reaction mechanism it is important to study more crystal structures of cineole synthases because although certain monoterpene synthases synthesize the same product they do not share sequence similarities. Therefore it might not be a question to know the exact amino acids involved in the active pocket but rather which characteristics and properties of amino acids support a certain reaction mechanism. Another approach to elucidate the reaction mechanism for the formation of 1,8-cineole could be the analysis of kinetic isotope effects. This is one of the most important methods to analyze different reaction mechanisms. Gatto et al. (2015) used kinetic isotope effects to analyze the multi-product synthases from *Zea mays*. They used mono- or hexadeutered geranyl diphosphate or farnesyl diphosphate and incubated these substrates with the sesquiterpene synthases from *Z. mays* to find out branching points in complex cyclization reaction (Gatto et al. 2015). Similar deuterium labelling studies could be also used to analyze the reaction mechanism to 1,8-cineole. Another idea to find out what amino acids influence the product distribution could be the comparison of the single-product 1,8-cineole synthase from *C. unshiu* (Shimada et al. 2005) with the multi-product 1,8-cineole synthase of *N. suaveolens*. If substitutions of different amino acids in the multi-product cineole synthase would reveal mutant enzymes which synthesize only 1,8-cineole the synthesis of 1,8-cineole via pathway B (figure 35) could be assumed.

An additional important experiment would be the analysis of interesting mutant enzymes and also of the wild type enzyme *in vivo*. All analyses about the mutant enzymes were done *in vitro* by heterologous overexpression in *E. coli*. It would be also essential to study the effect of the mutant enzymes and also of the wild type enzyme *in planta*. Cloning of the mutant enzyme F266S in a binary plant vector system to transform this construct in *A. thaliana* could be done. *A. thaliana* is a good investigated model plant and own investigations of the emitted volatiles by the flowers and leaves showed that *A. thaliana* did not emit any of the monoterpenes investigated in this study. But it has to be considered that Chen et al. (2004) isolated a 1,8-cineole synthase from the roots.

The analysis of the mutant enzymes and wild type enzyme *in vivo* of the species *N. suaveolens* would be optimal. 1,8-cineole synthase knock-out mutants of *N. suaveolens* should be complemented with the wild type enzyme

as well as mutant enzymes. These experiments could verify the *in vitro* product profiles and subsequently the assumed mechanism and involved amino acids. The *in planta* analysis could also give insights into ecological aspects of monoterpenes in plants. Do the emitted monoterpenes protect plants against herbivory or are they important to attract pollinators? Most of the monoterpenoid compounds have different antifungal and antimicrobial functions (Jirovetz et al. 2006). This underlines the hypothesis of protection. Most of the emitted monoterpenoids were found in the petals which would support the hypothesis of attraction of pollinators. However, Pichersky et al. (1994) investigated low levels of monoterpene emission and linalool synthase activity in the stigma of *Clarkia concinna* and suggested an additional function. Therefore further experiments should be done to elucidate the function of the multiple synthesized monoterpenoids.

Nicotiana species are pollinated by several pollinators (e. g. bees, hawkmoth, hummingbird). It might be interesting to analyze the effect of the mutant 1,8-cineole synthases e. g. the mutant F266S concerning the volatile profile and in general the effect of the α-terpineol/1,8-cineole ratio on the pollinator behavior. One approach to analyze the behavior of pollinators towards the emitted plant volatiles could be the employment of a flight tunnel.

7. List of literature

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8. Supplement

Supplement 1

Table S1: Mutated primer pairs for site-directed mutagenesis to generate *E. coli* mutants. The table shows the name of the primer pair, the sequence from 5 → 3 and in red marked the mutation.

name of the primer	sequence of the primer
ClNsuav G7Q TERlang	fw 5'→3': CGTTCAGGAAATTACCAACCTACCATGTGGG rv 5'→3': CCCACATGGTAGGTTGGTAATTTCCTGAACG
ClNsuav C235G TERlang	fw 5'→3': GTGGTGGAAAGAAACAGGCTTAGCAGAGAATCTG rv 5'→3': CAGATTCTCTGCTAAGCCTGTTTCTTTCCACCAC
ClNsuav F266S TERlang	fw 5'→3': CTTCCTCAATATGGATACTCCAGAAGAATTGAGACGAAG rv 5'→3': CTTCGTCTCAATTCTTCTGGAGTATCCATATTGAGGAAG
ClNsuav F266Y TERlang	fw 5'→3': CCTTCCTCAATATGGATACTACAGAAGAATTGAGACGAAG rv 5'→3': CTTCGTCTCAATTCTTCTGTAGTATCCATATTGAGGAAGG
ClNsuav F266Thr TERlang	fw 5'→3': CTCAATATGGATACACAAGAAGAATTGAGACGAAGG rv 5'→3': CCTTCGTCTCAATTCTTCTTGTGTATCCATATTGAG
ClNsuav F266C TERlang	fw 5'→3': CTTCCTCAATATGGATACTGTAGAAGAATTGAGACGAAGG rv 5'→3': CCTTCGTCTCAATTCTTCTACAGTATCCATATTGAGGAAG
ClNsuav F266V TERlang	fw 5'→3': CCTTCCTCAATATGGATACGTGAGAAGAATTGAGACGAAGG rv 5'→3': CCTTCGTCTCAATTCTTCTCACGTATCCATATTGAGGAAGG
ClNsuav A279T TERlang	fw 5'→3': GTAAATGCTTTAGTAACCGCAATAGACGATGTATATG

	rv 5‘→3‘: CATATACATCGTCTATTGCGGTTACTAAAGCATTTAC
M290L	fw 5‘→3‘: P-GATGTTTTTGGTACACTGGATGAACTACAATGC rv 5‘→3‘: P-GCATTGTAGTTCATCCAGTGTACCAAAAACATC
H298D	fw 5‘→3‘: GAACTACAATGCTTCACTGATGCCTTTCAAAGATGG rv 5‘→3‘: CCATCTTTGAAAGGCATCAGTGAAGCATTGTAGTTC
F300I	fw 5‘→3‘: CTACAATGCTTCACTCATGCCATTCAAAGATGGAATATCGACG rv 5‘→3‘: CGTCGATATTCCATCTTTGAATGGCATGAGTGAAGCATTGTAG
I305T	fw 5‘→3‘: P-CCTTTCAAAGATGGAATACCGACGAGCTGGATAAC rv 5‘→3‘: P-GTTATCCAGCTCGTCGGTATTCCATCTTTGAAAGGC
N324D	fw 5‘→3‘:TATTTTGCACTTGACGACTTTATTAATGAAGTG7 rv 5‘→3‘:CACTTCATTAATAAAGTCGTCAAGTGCAAAATA
ClNsuav delete E335E336	fw 5‘→3‘: TTTATTAATGAAGTGGCTGGTGATGCTTTT------ CACCGAGTTCCCA rv 5‘→3‘: TGGGAACTCGGTG------ AAAAGCATCACCAGCCACTTCATTAATAAA
A355S	fw 5‘→3‘: P-GATTTGTGCAAATCTTACTTAAGAGAAGCAAAG rv 5‘→3‘: P-CTTTGCTTCTCTTAAGTAAGATTTGCACAAATC
ClNsuav Y446A TERlang	fw 5‘→3‘: GTACCCAAATCAATACAATGTGCAATGAACGAAAAGAAAGTTTC rv 5‘→3‘: GAAACTTTCTTTTCGTTCATTGCACATTGTATTGATTTGGGTAC
ClNsuav Y502V TERlang	fw 5‘→3‘:CAAGAATGGCACAATGCATGGTACAGCATGGTGATGGACATG rv 5‘→3‘:CATGTCCATCACCATGCTGTACCATGCATTGTGCCATTCTTG

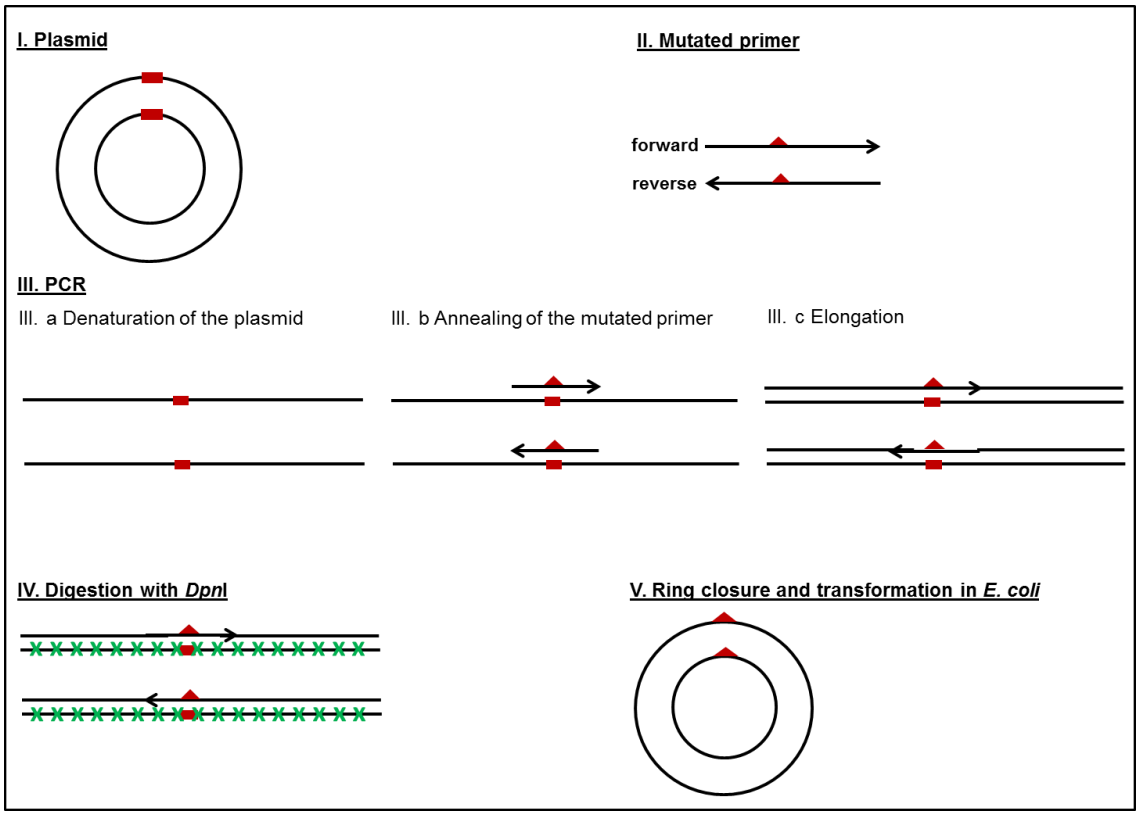


Figure S1: Schematic process of the method: site-directed mutagenesis.
The double-stranded plasmid could be mutated in an optional segment of the gene (I). The generated primer pairs (forward und reverse) carry the mutation (II). Towards denaturation (III. a) of the double-stranded plasmid the primer could bind on the respective strand (III. b). During elongation (III. c) the primer was amplified and the new synthesized double-strand carried the specific mutation. The parental plasmid is digested by the restriction enzyme *Dpn*I (IV). The ring closure of the new synthesized plasmid is realized during the transformation in *E. coli* (V).

Approach for the mutagenesis PCR and the program for the thermocycler (Thermo Scientific™ Hybaid, Fisher Scientific):

PCR approach:

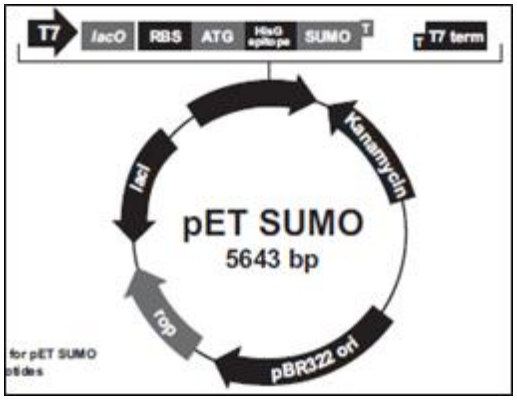
∑= 25 µl	
2,5 µl	10 x *PfuTurbo* Puffer (Agilent Technologies)
1,5 µl	Primer forward (10 pmol, Invitrogen)
1,5 µl	Primer reverse (10 pmol, Invitrogen)
1 µl	dNTPs (10 mM each, Fermentas)
0,5 µl	Plasmid
17,5 µl	Aqua dest.
0,5 µl	*PfuTurbo*® DNA-Polymerase (Agilent Technologies)

PCR program:

97 °C	2 min	
97 °C	50 sec	18 cycles
55 °C	50 sec	
68 °C	2 min/Kbp	
68 °C	10 min	
10 °C	hold	

The elongation time was increased to 2 min/kbp to ensure that the whole plasmid was amplified.

Vector map: Champion^{TM} pET Sumo Protein Vektor (Invitrogen):



Supplement 2

In the following supplement 2 are listed the complete sequences from the N-terminal RR$(X)_8$W motif to the stop. The alignments of the wild type 1,8-cineole synthase of *N. suaveolens* (CINsuavWT) and each mutant enzyme were created with CLUSTALW 2.0.10 (Copyright by Des Higgins, Julie Thompson and Toby Gibson). The red letters show the amino acids changed in the sequence and in the alignment.

mutant G7Q

sequence:

RRSGNY**Q**PTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
G7Qclone1       RRSGNYQPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ****** **************************************************

G7Qclone1       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

G7Qclone1       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

G7Qclone1       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

G7Qclone1       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

G7Qclone1       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

G7Qclone1       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

G7Qclone1       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

G7Qclone1       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ****************************************************
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mutant C235G

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
GLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
C235Gclone2     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

C235Gclone2     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

C235Gclone2     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

C235Gclone2     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETGLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ********************************************************* *****

C235Gclone2     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

C235Gclone2     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

C235Gclone2     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

C235Gclone2     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

C235Gclone2     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ****************************************************
```

mutant F266S

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGY**S**RRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F266Sclone7     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

F266Sclone7     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

F266Sclone7     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

F266Sclone7     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

F266Sclone7     PFARDRLVENFFWTIGVNFLPQYGYSRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************ ********************************

F266Sclone7     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

F266Sclone7     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

F266Sclone7     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

F266Sclone7      HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ****************************************************
```

mutant F266C

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGY**C**RRIETKVNALVTTIDDVYDVFGTMDE
LQCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAW
TDLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLE
NYHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F266Cclone11     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT        RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                 ************************************************************

F266Cclone11     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT        QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                 ************************************************************

F266Cclone11     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT        EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                 ************************************************************

F266Cclone11     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT        WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                 ************************************************************

F266Cclone11     PFARDRLVENFFWTIGVNFLPQYGYCRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT        PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                 ************************ ********************************

F266Cclone11     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT        QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                 ************************************************************

F266Cclone11     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT        KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                 ************************************************************

F266Cclone11     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT        LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                 ************************************************************

F266Cclone11     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT        HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                 ****************************************************
```

mutant F266Y

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGY**Y**RRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F266Yclone5     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

F266Yclone5     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

F266Yclone5     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

F266Yclone5     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

F266Yclone5     PFARDRLVENFFWTIGVNFLPQYGYYRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************:***********************************

F266Yclone5     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

F266Yclone5     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

F266Yclone5     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

F266Yclone5     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant F266T

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGY**T**RRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F266Tclone21          RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT             RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                      ************************************************************

F266Tclone21          QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT             QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                      ************************************************************

F266Tclone21          EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT             EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                      ************************************************************

F266Tclone21          WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT             WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                      ************************************************************

F266Tclone21          PFARDRLVENFFWTIGVNFLPQYGYTRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT             PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                      ************************ ********************************

F266Tclone21          QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT             QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                      ************************************************************

F266Tclone21          KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT             KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                      ************************************************************

F266Tclone21          LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT             LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                      ************************************************************

F266Tclone21          HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT             HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                      ****************************************************
```

mutant F266V

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGY**V**RRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F266Vclone14     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT        RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                 ************************************************************

F266Vclone14     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT        QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                 ************************************************************

F266Vclone14     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT        EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                 ************************************************************

F266Vclone14     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT        WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                 ************************************************************

F266Vclone14     PFARDRLVENFFWTIGVNFLPQYGYVRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT        PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                 ************************.***********************************

F266Vclone14     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT        QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                 ************************************************************

F266Vclone14     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT        KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                 ************************************************************

F266Vclone14     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT        LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                 ************************************************************

F266Vclone14     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT        HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                 ************************************************** 
```

mutant G7Q/M290L

sequence:

RRSGNY**Q**PTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGT**L**DEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
G7Q/M290Lclone3     RRSGNYQPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT           RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                    ****** **************************************************

G7Q/M290Lclone3     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT           QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                    ************************************************************

G7Q/M290Lclone3     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT           EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                    ************************************************************

G7Q/M290Lclone3     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT           WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                    ************************************************************

G7Q/M290Lclone3     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTLDELQCFTHAF 300
CINsuavWT           PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                    ************************************************:***********

G7Q/M290Lclone3     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT           QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                    ************************************************************

G7Q/M290Lclone3     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT           KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                    ************************************************************

G7Q/M290Lclone3     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT           LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                    ************************************************************

G7Q/M290Lclone3     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT           HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                    ****************************************************
```

mutant T279A

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVT**A**IDDVYDVFGTMDE
LQCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAW
TDLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLE
NYHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
T279Aclone1     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

T279Aclone1     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

T279Aclone1     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

T279Aclone1     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

T279Aclone1     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTAIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************:***********************

T279Aclone1     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

T279Aclone1     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

T279Aclone1     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

T279Aclone1     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ****************************************************
```

mutant M290L

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGT**L**DEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
M290Lclone4     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
M290Lclone4     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
M290Lclone4     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
M290Lclone4     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
M290Lclone4     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTLDELQCFTHAF 300
                ************************************************:***********

CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
M290Lclone4     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
M290Lclone4     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
M290Lclone4     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
M290Lclone4     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant H298D

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFT**D**AFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
H298Dclone1     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

H298Dclone1     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

H298Dclone1     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

H298Dclone1     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

H298Dclone1     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTDAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************.**

H298Dclone1     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

H298Dclone1     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

H298Dclone1     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

H298Dclone1     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant M290L/H298D

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGT**L**DEL
QCFT**D**AFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
M290LH298Dclone2     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT            RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                     ************************************************************

M290LH298Dclone2     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT            QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                     ************************************************************

M290LH298Dclone2     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT            EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                     ************************************************************

M290LH298Dclone2     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT            WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                     ************************************************************

M290LH298Dclone2     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTLDELQCFTDAF 300
CINsuavWT            PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                     ************************************************:*******.**

M290LH298Dclone2     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT            QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                     ************************************************************

M290LH298Dclone2     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT            KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                     ************************************************************

M290LH298Dclone2     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT            LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                     ************************************************************

M290LH298Dclone2     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT            HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                     **************************************************
```

mutant F300I

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHA**I**QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTD
LCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENY
HDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETW
KKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALL
FESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
F300Iclone1     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

F300Iclone1     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************


F300Iclone1     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

F300Iclone1     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

F300Iclone1     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAI 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************:

F300Iclone1     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

F300Iclone1     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

F300Iclone1     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

F300Iclone1     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant I305T

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNTDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
I305Tclone2     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

I305Tclone2     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

I305Tclone2     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

I305Tclone2     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

I305Tclone2     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

I305Tclone2     QRWNTDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                **** ********************************************************

I305Tclone2     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

I305Tclone2     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

I305Tclone2     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ******************************************************
```

mutant N324D

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALD**D**FINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
N324Dclone4     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

N324Dclone4     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

N324Dclone4     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

N324Dclone4     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

N324Dclone4     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

N324Dclone4     QRWNIDELDNLPDNMKMCYFALDDFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                **********************:*************************************

N324Dclone4     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

N324Dclone4     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

N324Dclone4     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant EE335/336-deleted

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAF(EE)HRVPILSYLRNAW
TDLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLE
NYHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
EE335/336deletedclone2   RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT                RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                         ************************************************************

EE335/336deletedclone2   QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT                QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                         ************************************************************

EE335/336deletedclone2   EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT                EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                         ************************************************************

EE335/336deletedclone2   WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT                WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                         ************************************************************

EE335/336deletedclone2   PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT                PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                         ************************************************************

EE335/336deletedclone2   QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAF--HRVPILSYLRNAWTDLCKAYLREA 358
CINsuavWT                QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                         ********************************  ************************

EE335/336deletedclone2   KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 418
CINsuavWT                KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                         ************************************************************

EE335/336deletedclone2   LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 478
CINsuavWT                LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                         ************************************************************

EE335/336deletedclone2   HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 530
CINsuavWT                HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                         ****************************************************
```

mutant A355S

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCK**S**YLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
A355Sclone3     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

A355Sclone3     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

A355Sclone3     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

A355Sclone3     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

A355Sclone3     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

A355Sclone3     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKSYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************:*****

A355Sclone3     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

A355Sclone3     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

A355Sclone3     HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                **************************************************
```

mutant Y446A

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQC**A**MNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
Y446Aclone15    RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
WTCINsuav       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

Y446Aclone15    QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
WTCINsuav       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

Y446Aclone15    EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
WTCINsuav       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

Y446Aclone15    WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
WTCINsuav       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

Y446Aclone15    PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
WTCINsuav       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

Y446Aclone15    QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
WTCINsuav       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

Y446Aclone15    KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
WTCINsuav       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

Y446Aclone15    LRLANDLGTSSEELKRGDVPKSIQCAMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
WTCINsuav       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************ ********************************

Y446Aclone15    HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
WTCINsuav       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ****************************************************
```

mutant Y502V

sequence:

RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELI
DNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSE
THTEDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHA
LELPLHWRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKET
CLAENLPFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDEL
QCFTHAFQRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWT
DLCKAYLREAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLEN
YHDIIRWSALILRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISET
WKKLNEAHNVAAHPFPKMFVKCAMNLARMAQCM**V**QHGDGHGHGDLNSETTNHIMA
LLFESIPPA

alignment with the wild type enzyme (CINsuavWT):

```
Y502Vclone4     RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
CINsuavWT       RRSGNYGPTMWDFEYIQSIHNDYTEKKYMNRLNKLKEEMKKMIMAEGSQELEKLELIDNL 60
                ************************************************************

Y502Vclone4     QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
CINsuavWT       QRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLFREYGFHISQEIFGGLSETHT 120
                ************************************************************

Y502Vclone4     EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
CINsuavWT       EDTKGMLYLYEASFLATEGESELEKARNWTEKHLREYLENKNDDQNVAELVHHALELPLH 180
                ************************************************************

Y502Vclone4     WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
CINsuavWT       WRMLRIEARWFINFYKKKQDMIPLLLELAILDFNIVQAAHIEDLKYVARWWKETCLAENL 240
                ************************************************************

Y502Vclone4     PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
CINsuavWT       PFARDRLVENFFWTIGVNFLPQYGYFRRIETKVNALVTTIDDVYDVFGTMDELQCFTHAF 300
                ************************************************************

Y502Vclone4     QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
CINsuavWT       QRWNIDELDNLPDNMKMCYFALDNFINEVAGDAFEEHRVPILSYLRNAWTDLCKAYLREA 360
                ************************************************************

Y502Vclone4     KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
CINsuavWT       KWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLVANPVNKEVLHYLENYHDIIRWSALI 420
                ************************************************************

Y502Vclone4     LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
CINsuavWT       LRLANDLGTSSEELKRGDVPKSIQCYMNEKKVSEEEARQHIRLLISETWKKLNEAHNVAA 480
                ************************************************************

Y502Vclone4     HPFPKMFVKCAMNLARMAQCMVQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
CINsuavWT       HPFPKMFVKCAMNLARMAQCMYQHGDGHGHGDLNSETTNHIMALLFESIPPA 532
                ******************* ****************************
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Supplement 3

The alignment of all 19 nucleotide sequences of the mutant enzymes with the wild type 1,8-cineole synthase from *N. suaveolens* (CINsuavWT) was created with CLUSTALW 2.0.10 (Copyright by Des Higgins, Julie Thompson and Toby Gibson). The red marked letters show the nucleotide alterations.

```
CINsuavWT            AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
EE335/336deleted     AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
A355S                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
N324D                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
C235G                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
I305T                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F300I                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
T279A                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
Y446A                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
Y502V                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F266T                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F266V                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F266C                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F266S                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
F266Y                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
G7Q                  AGACGTTCAGGAAATTACCAACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
G7Q/M290L            AGACGTTCAGGAAATTACCAACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
M290L                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
H298D                AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
M290L/H298D          AGACGTTCAGGAAATTACGGACCTACCATGTGGGATTTTGAGTATATTCAGTCCATACAC 60
                     ******************  ************************************

CINsuavWT            AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
EE335/336deleted     AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
A355S                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
N324D                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
C235G                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
I305T                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F300I                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
T279A                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
Y446A                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
Y502V                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F266T                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F266V                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F266C                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F266S                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
F266Y                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
G7Q                  AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
G7Q/M290L            AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
M290L                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
H298D                AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
M290L/H298D          AATGATTATACGGAAAAGAAGTATATGAATCGTTTAAACAAACTGAAGGAGGAAATGAAG 120
                     ************************************************************

CINsuavWT            AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
EE335/336deleted     AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
A355S                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
N324D                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
C235G                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
I305T                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F300I                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
T279A                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
Y446A                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
Y502V                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F266T                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F266V                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F266C                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F266S                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
F266Y                AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
G7Q                  AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
G7Q/M290L            AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
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M290L               AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
H298D               AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
M290L/H298D         AAGATGATAATGGCCGAGGGATCACAAGAGTTAGAGAAGTTGGAGCTGATTGATAATTTA 180
                    ************************************************************

CINsuavWT           CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
EE335/336deleted    CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
A355S               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
N324D               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
C235G               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
I305T               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F300I               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
T279A               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
Y446A               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
Y502V               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F266T               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F266V               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F266C               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F266S               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
F266Y               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
G7Q                 CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
G7Q/M290L           CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
M290L               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
H298D               CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
M290L/H298D         CAAAGGCTTGGAGTTAGTTACCACTTCAAGCATGAAATTATGCAAATTTTGAGCAGCATA 240
                    ************************************************************

CINsuavWT           AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
EE335/336deleted    AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
A355S               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
N324D               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
C235G               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
I305T               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F300I               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
T279A               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
Y446A               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
Y502V               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F266T               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F266V               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F266C               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F266S               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
F266Y               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
G7Q                 AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
G7Q/M290L           AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
M290L               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
H298D               AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
M290L/H298D         AAGCAGCACAGTACTCCAGCAGACTCATTATACGCCACAGCTCTGAAATTTAGACTCTTT 300
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CINsuavWT           AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
EE335/336deleted    AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
A355S               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
N324D               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
C235G               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
I305T               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F300I               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
T279A               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
Y446A               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
Y502V               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F266T               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F266V               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F266C               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F266S               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
F266Y               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
G7Q                 AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
G7Q/M290L           AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
M290L               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
H298D               AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
M290L/H298D         AGAGAATATGGTTTTCATATCTCTCAAGAAATATTCGGTGGTCTTAGTGAAACTCATACT 360
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CINsuavWT           GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
EE335/336deleted    GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
A355S               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
N324D               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
C235G               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
I305T               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
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F300I               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
T279A               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
Y446A               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
Y502V               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
F266T               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
F266V               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
F266C               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
F266S               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
F266Y               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
G7Q                 GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
G7Q/M290L           GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
M290L               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
H298D               GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
M290L/H298D         GAGGATACAAAGGGGATGCTATATTTGTATGAAGCTTCTTTTCTTGCAACAGAAGGTGAG 420
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CINsuavWT           AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
EE335/336deleted    AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
A355S               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
N324D               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
C235G               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
I305T               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F300I               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
T279A               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
Y446A               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
Y502V               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F266T               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F266V               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F266C               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F266S               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
F266Y               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
G7Q                 AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
G7Q/M290L           AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
M290L               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
H298D               AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
M290L/H298D         AGTGAATTGGAGAAAGCAAGAAATTGGACAGAAAAACACTTAAGAGAATATTTGGAGAAT 480
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CINsuavWT           AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
EE335/336deleted     AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
A355S               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
N324D               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
C235G               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
I305T               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F300I               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
T279A               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
Y446A               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
Y502V               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F266T               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F266V               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F266C               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F266S               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
F266Y               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
G7Q                 AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
G7Q/M290L           AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
M290L               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
H298D               AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
M290L/H298D         AAAAATGATGACCAAAATGTGGCTGAATTAGTGCACCATGCTTTGGAACTTCCATTGCAT 540
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CINsuavWT           TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
EE335/336deleted    TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
A355S               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
N324D               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
C235G               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
I305T               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F300I               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
T279A               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
Y446A               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
Y502V               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F266T               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F266V               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F266C               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F266S               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
F266Y               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
G7Q                 TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
G7Q/M290L           TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
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M290L               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
H298D               TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
M290L/H298D         TGGAGGATGTTGAGAATAGAGGCAAGGTGGTTCATCAATTTTTACAAGAAAAAACAAGAC 600
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CINsuavWT           ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
EE335/336deleted    ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
A355S               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
N324D               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
C235G               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
I305T               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F300I               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
T279A               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
Y446A               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
Y502V               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F266T               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F266V               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F266C               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F266S               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
F266Y               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
G7Q                 ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
G7Q/M290L           ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
M290L               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
H298D               ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
M290L/H298D         ATGATTCCTTTATTGCTTGAGCTGGCTATACTTGACTTCAACATTGTTCAAGCAGCTCAT 660
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CINsuavWT           ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
EE335/336deleted    ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
A355S               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
N324D               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
C235G               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACAGGCTTAGCAGAGAATCTG 720
I305T               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F300I               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
T279A               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
Y446A               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
Y502V               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F266T               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F266V               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F266C               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F266S               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
F266Y               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
G7Q                 ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
G7Q/M290L           ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
M290L               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
H298D               ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
M290L/H298D         ATTGAAGACCTAAAATATGTGGCAAGGTGGTGGAAAGAAACATGCTTAGCAGAGAATCTG 720
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CINsuavWT           CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
EE335/336deleted    CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
A355S               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
N324D               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
C235G               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
I305T               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F300I               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
T279A               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
Y446A               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
Y502V               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F266T               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F266V               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F266C               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F266S               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
F266Y               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
G7Q                 CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
G7Q/M290L           CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
M290L               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
H298D               CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
M290L/H298D         CCATTTGCTAGGGATAGATTAGTGGAGAATTTCTTTTGGACAATTGGAGTAAATTTCCTT 780
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CINsuavWT           CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
EE335/336deleted    CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
A355S               CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
N324D               CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
C235G               CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
I305T               CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
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F300I                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
T279A                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCGCAATA 840
Y446A                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
Y502V                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
F266T                CCTCAATATGGATACACAAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
F266V                CCTCAATATGGATACGTGAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
F266C                CCTCAATATGGATACTGTAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
F266S                CCTCAATATGGATACTCCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
F266Y                CCTCAATATGGATACTACAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
G7Q                  CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
G7Q/M290L            CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
M290L                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
H298D                CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
M290L/H298D          CCTCAATATGGATACTTCAGAAGAATTGAGACGAAGGTAAATGCTTTAGTAACCACAATA 840
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CINsuavWT            GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
EE335/336deleted     GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
A355S                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
N324D                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
C235G                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
I305T                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F300I                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCATT 900
T279A                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
Y446A                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
Y502V                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F266T                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F266V                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F266C                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F266S                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
F266Y                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
G7Q                  GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTCATGCCTTT 900
G7Q/M290L            GACGATGTATATGATGTTTTTGGTACACTGGATGAACTACAATGCTTCACTCATGCCTTT 900
M290L                GACGATGTATATGATGTTTTTGGTACACTGGATGAACTACAATGCTTCACTCATGCCTTT 900
H298D                GACGATGTATATGATGTTTTTGGTACAATGGATGAACTACAATGCTTCACTGATGCCTTT 900
M290L/H298D          GACGATGTATATGATGTTTTTGGTACACTGGATGAACTACAATGCTTCACTGATGCCTTT 900
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CINsuavWT            CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
EE335/336deleted     CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
A355S                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
N324D                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
C235G                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
I305T                CAAAGATGGAATACCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F300I                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
T279A                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
Y446A                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
Y502V                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F266T                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F266V                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F266C                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F266S                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
F266Y                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
G7Q                  CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
G7Q/M290L            CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
M290L                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
H298D                CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
M290L/H298D          CAAAGATGGAATATCGACGAGCTGGATAACCTCCCAGACAATATGAAGATGTGTTATTTT 960
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CINsuavWT            GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
EE335/336deleted     GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTT------CACCGAGTTCCC 1014
A355S                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
N324D                GCACTTGACGACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
C235G                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
I305T                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F300I                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
T279A                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
Y446A                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
Y502V                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F266T                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F266V                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F266C                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F266S                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
F266Y                GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
G7Q                  GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
G7Q/M290L            GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
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M290L               GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
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M290L/H298D         GCACTTGACAACTTTATTAATGAAGTGGCTGGTGATGCTTTTGAAGAGCACCGAGTTCCC 1020
                    ********* ********************************     ************

CINsuavWT           ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
EE335/336deleted    ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1074
A355S               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAATCTTACTTAAGAGAAGCA 1080
N324D               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
C235G               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
I305T               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F300I               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
T279A               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
Y446A               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
Y502V               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F266T               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F266V               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F266C               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F266S               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
F266Y               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
G7Q                 ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
G7Q/M290L           ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
M290L               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
H298D               ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
M290L/H298D         ATTTTGTCCTATCTAAGAAATGCGTGGACAGATTTGTGCAAAGCTTACTTAAGAGAAGCA 1080
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CINsuavWT           AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
EE335/336deleted    AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1134
A355S               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
N324D               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
C235G               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
I305T               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F300I               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
T279A               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
Y446A               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
Y502V               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F266T               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F266V               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F266C               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F266S               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
F266Y               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
G7Q                 AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
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M290L               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
H298D               AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
M290L/H298D         AAGTGGTACTTTAGCAAGTATATTCCAACCATGGAAGAATACATGGACAATGCCTGGATT 1140
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EE335/336deleted    TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1194
A355S               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
N324D               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
C235G               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
I305T               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F300I               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
T279A               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
Y446A               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
Y502V               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F266T               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F266V               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F266C               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F266S               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
F266Y               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
G7Q                 TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
G7Q/M290L           TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
M290L               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
H298D               TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
M290L/H298D         TCGATATCAGCTCCAGTAATATTAGTACATGCCTATTTCCTCGTTGCTAACCCTGTAAAC 1200
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CINsuavWT           AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
EE335/336deleted    AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1254
A355S               AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
N324D               AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
C235G               AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
I305T               AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
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F300I                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
T279A                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
Y446A                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
Y502V                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
F266T                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
F266V                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
F266C                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
F266S                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
F266Y                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
G7Q                  AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
G7Q/M290L            AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
M290L                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
H298D                AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
M290L/H298D          AAGGAAGTTTTGCATTACTTAGAGAATTATCATGACATAATTCGTTGGTCAGCTTTGATT 1260
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CINsuavWT            TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
EE335/336deleted     TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1314
A355S                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
N324D                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
C235G                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
I305T                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F300I                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
T279A                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
Y446A                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
Y502V                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F266T                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F266V                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F266C                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F266S                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
F266Y                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
G7Q                  TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
G7Q/M290L            TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
M290L                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
H298D                TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
M290L/H298D          TTGCGGCTCGCAAATGATTTAGGAACATCATCGGAAGAGTTGAAAAGGGGTGATGTACCC 1320
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CINsuavWT            AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
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N324D                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
C235G                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
I305T                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F300I                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
T279A                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
Y446A                AAATCAATACAATGTGCAATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
Y502V                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F266T                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F266V                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F266C                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F266S                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
F266Y                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
G7Q                  AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
G7Q/M290L            AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
M290L                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
H298D                AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
M290L/H298D          AAATCAATACAATGTTACATGAACGAAAAGAAAGTTTCTGAAGAAGAGGCTCGACAACAT 1380
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CINsuavWT            ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
EE335/336deleted     ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1434
A355S                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
N324D                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
C235G                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
I305T                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F300I                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
T279A                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
Y446A                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
Y502V                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F266T                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F266V                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F266C                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F266S                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
F266Y                ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
G7Q                  ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
G7Q/M290L            ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
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M290L               ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
H298D               ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
M290L/H298D         ATTAGGCTTTTGATTAGTGAGACGTGGAAGAAATTGAACGAAGCTCATAATGTAGCAGCT 1440
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CINsuavWT           CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
EE335/336deleted    CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1494
A355S               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
N324D               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
C235G               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
I305T               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F300I               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
T279A               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
Y446A               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
Y502V               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F266T               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F266V               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F266C               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F266S               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
F266Y               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
G7Q                 CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
G7Q/M290L           CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
M290L               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
H298D               CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
M290L/H298D         CACCCCTTTCCTAAGATGTTTGTTAAGTGTGCTATGAACCTAGCAAGAATGGCACAATGC 1500
                    ************************************************************

CINsuavWT           ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
EE335/336deleted    ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1554
A355S               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
N324D               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
C235G               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
I305T               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F300I               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
T279A               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
Y446A               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
Y502V               ATGGTACAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F266T               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F266V               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F266C               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F266S               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
F266Y               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
G7Q                 ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
G7Q/M290L           ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
M290L               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
H298D               ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
M290L/H298D         ATGTATCAGCATGGTGATGGACATGGACATGGCGACCTGAATTCTGAGACAACAAATCAC 1560
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CINsuavWT           ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
EE335/336deleted    ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1593
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N324D               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
C235G               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
I305T               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F300I               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
T279A               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
Y446A               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
Y502V               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F266T               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F266V               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F266C               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F266S               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
F266Y               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
G7Q                 ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
G7Q/M290L           ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
M290L               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
H298D               ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
M290L/H298D         ATCATGGCATTGCTCTTTGAATCTATTCCTCCAGCTTGA 1599
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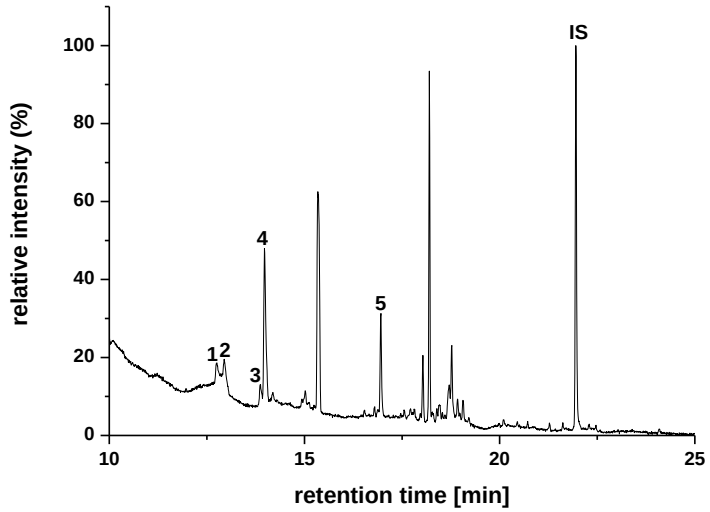
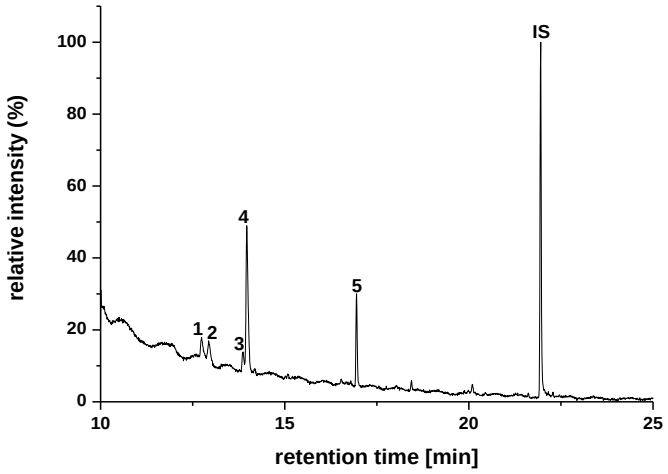
Supplement 4

In supplement 4 are shown the chromatograms of the wild type 1,8-cineole synthase and the mutant enzymes. Several chromatograms involve a red line. This reflects the result of a second experiment for the same mutant enzyme. X-axis = releative peak intensity (%), Y-axis = retention time [min]. 1: sabinene, 2: β-myrcene, 3: limonene, 4: 1,8-cineole, 5: α-terpineol, IS 1 and IS 2: internal Standard (cis-nerolidol). The internal standard was set 100 %.

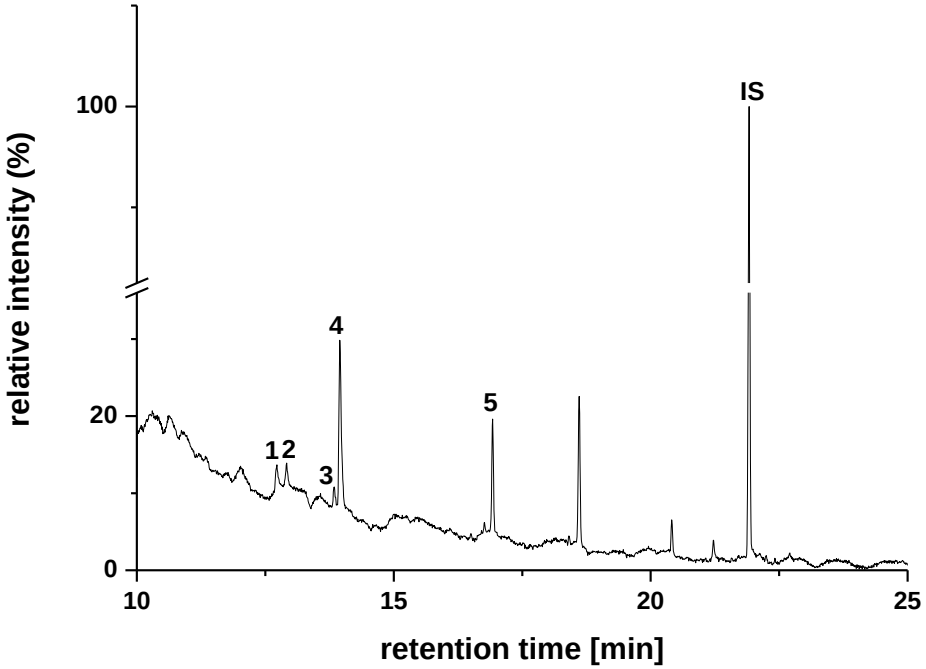
wild type enzyme, crude extract



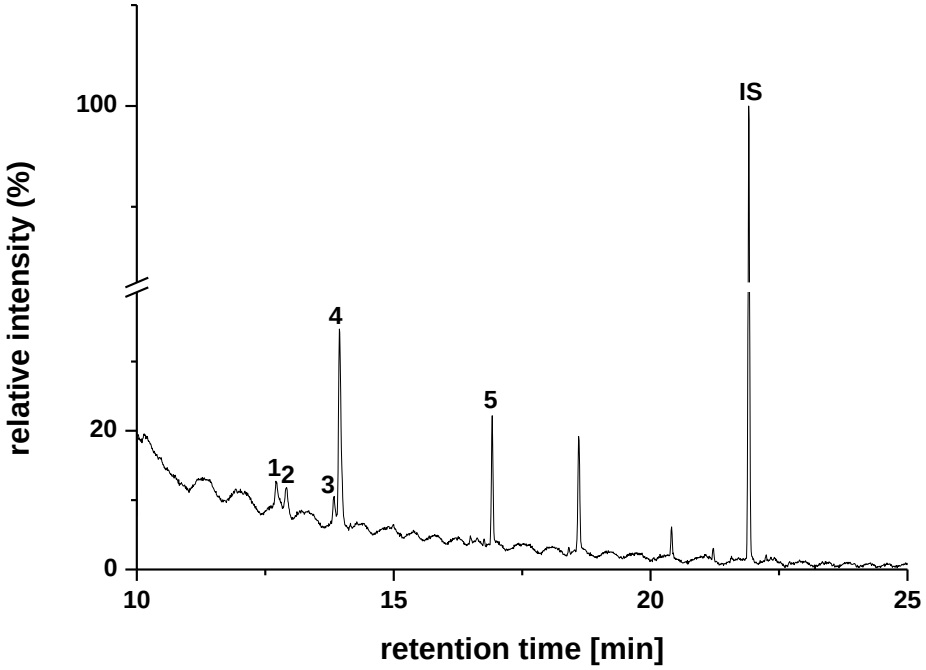
wild type enzyme, purified



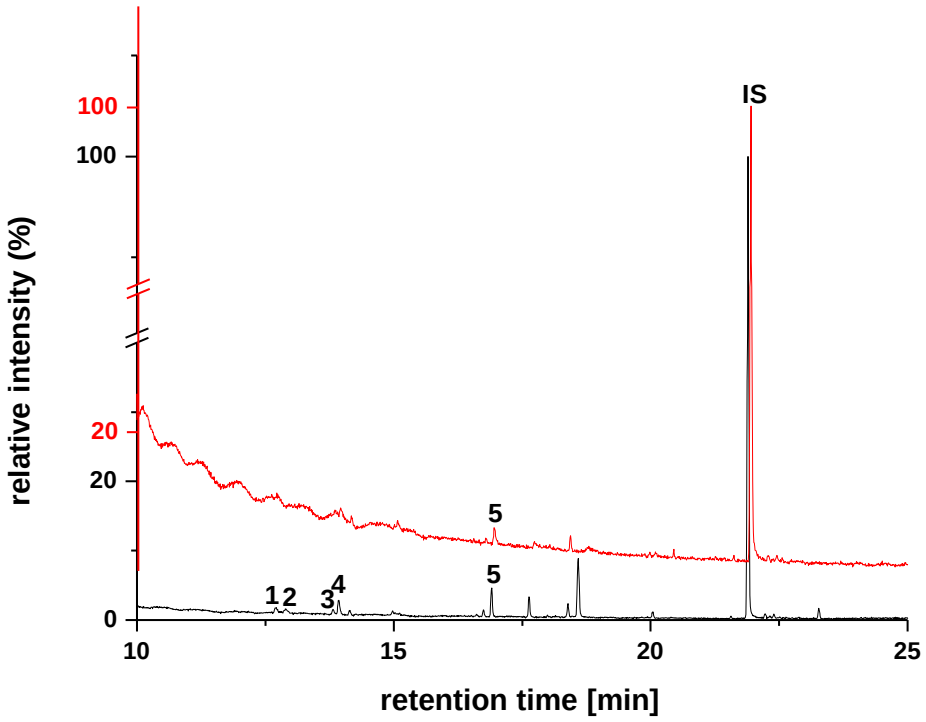
mutant G7Q, crude extract



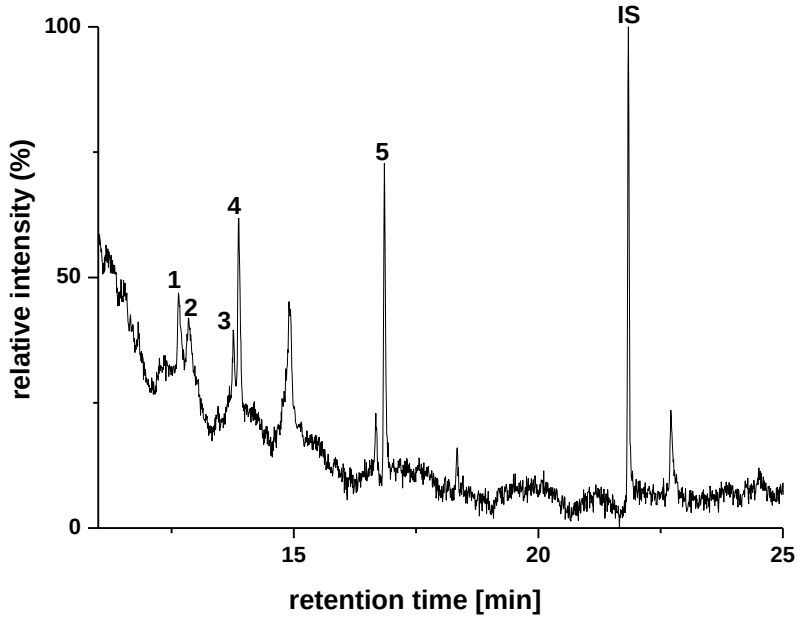
mutant C235G, crude extract



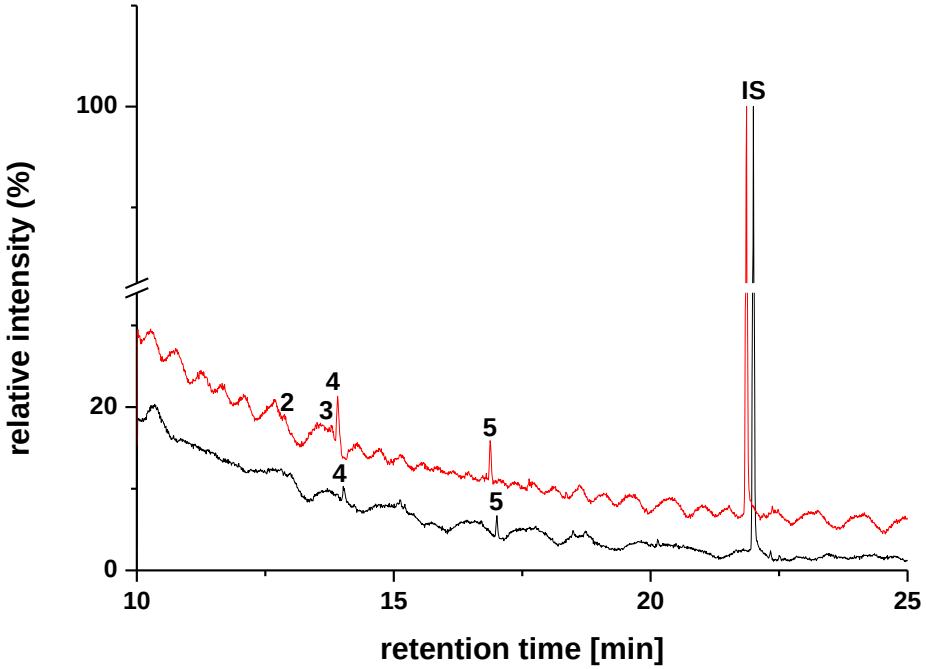
mutant F266S, crude extract



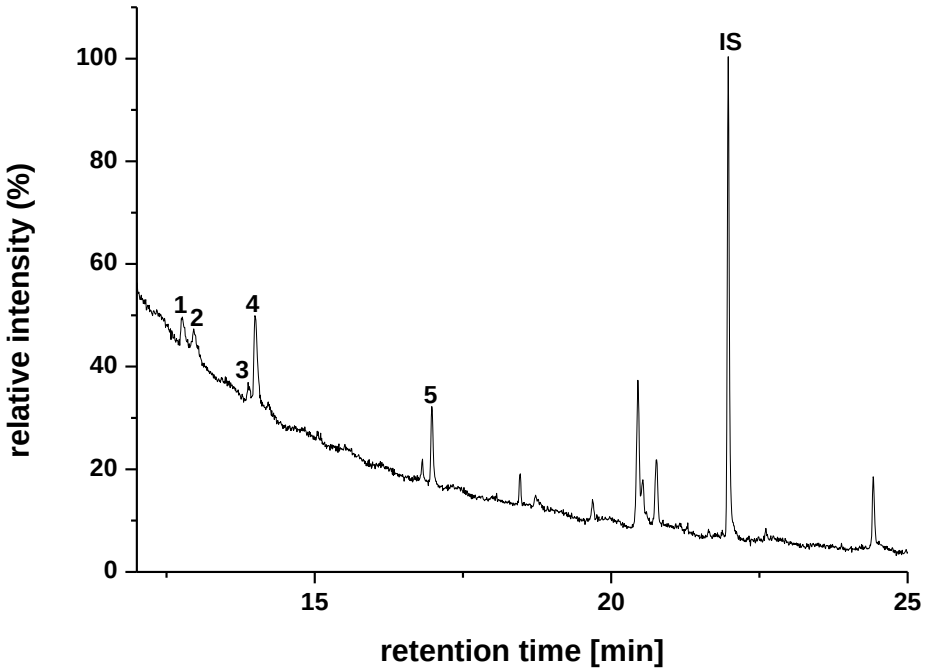
mutant F266S, purified



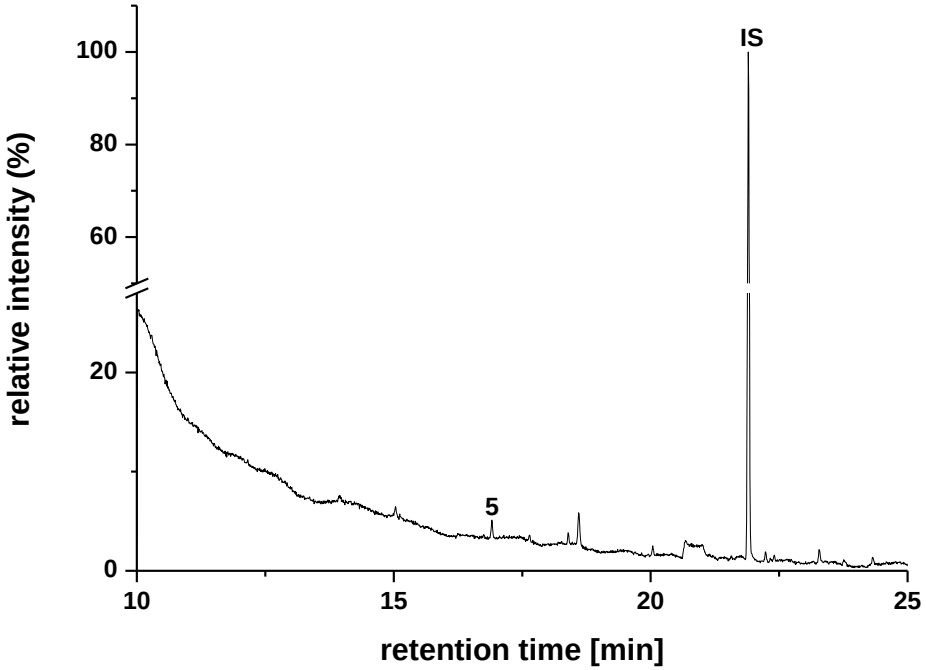
mutant F266C, crude extract



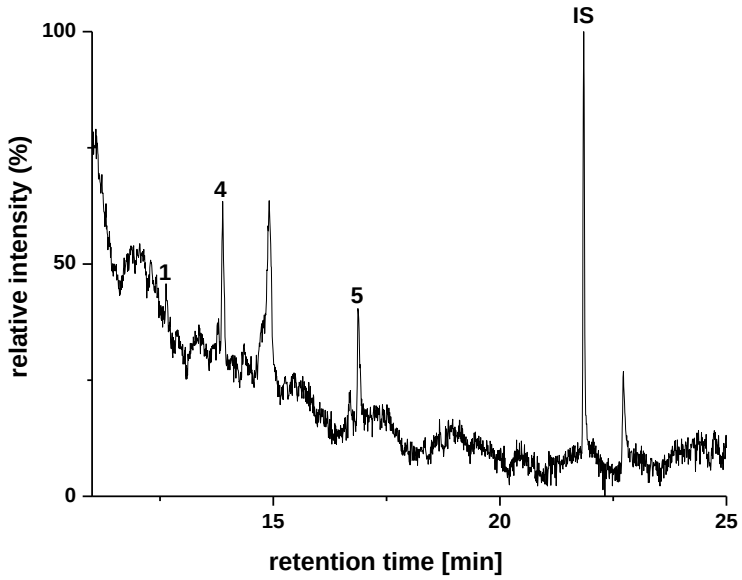
mutant F266Y, crude extract



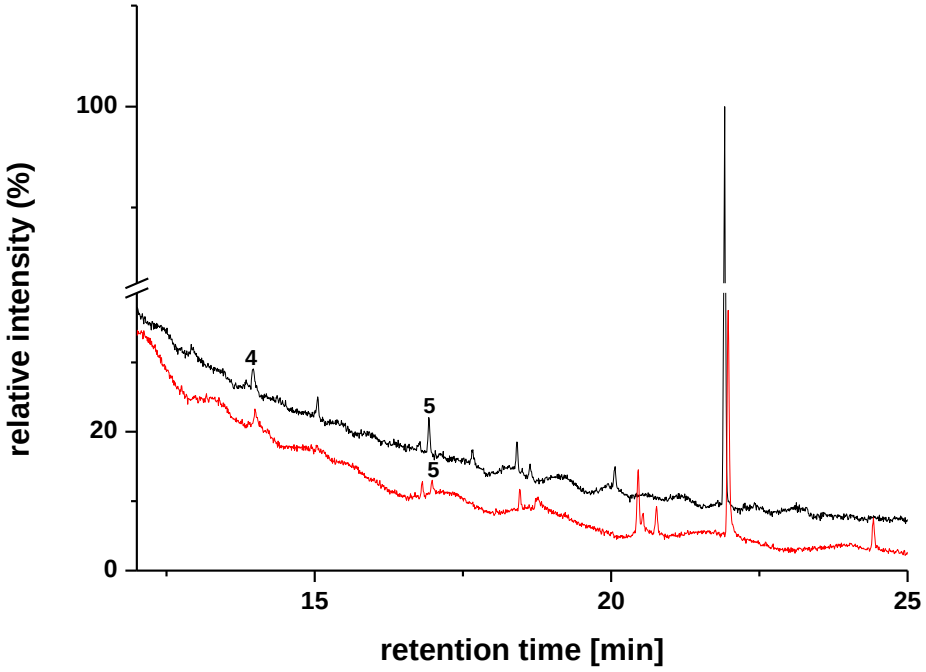
mutant F266T, crude extract



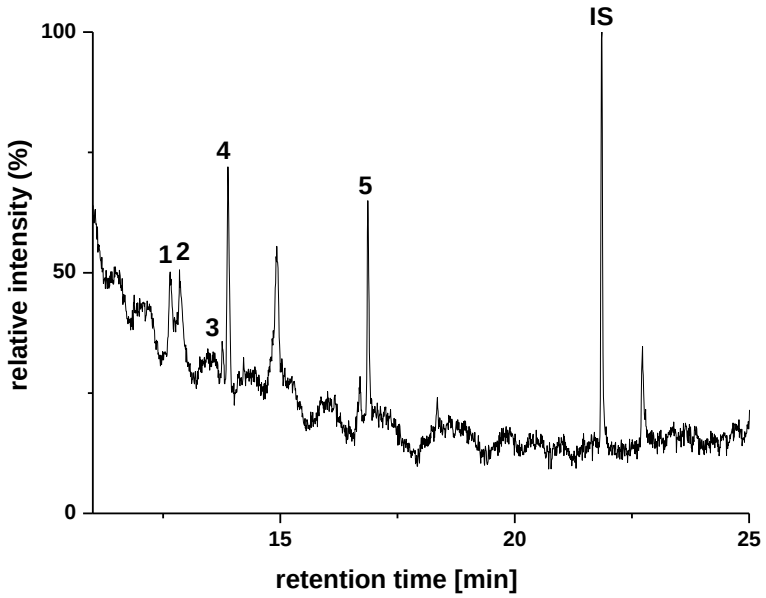
mutant F266T, purified



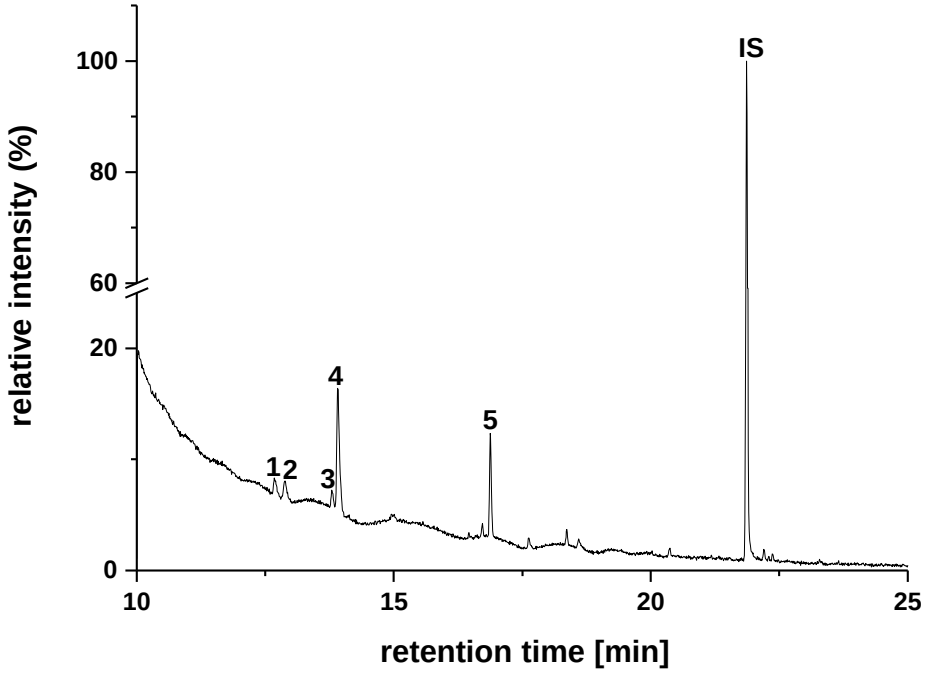
mutant F266V, crude extract



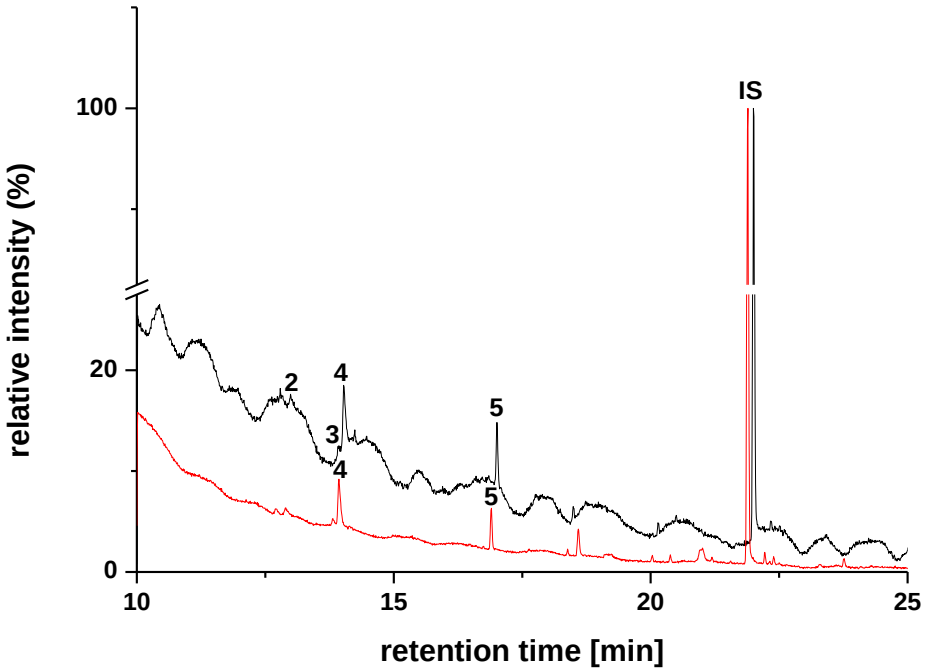
mutant F266V, purified



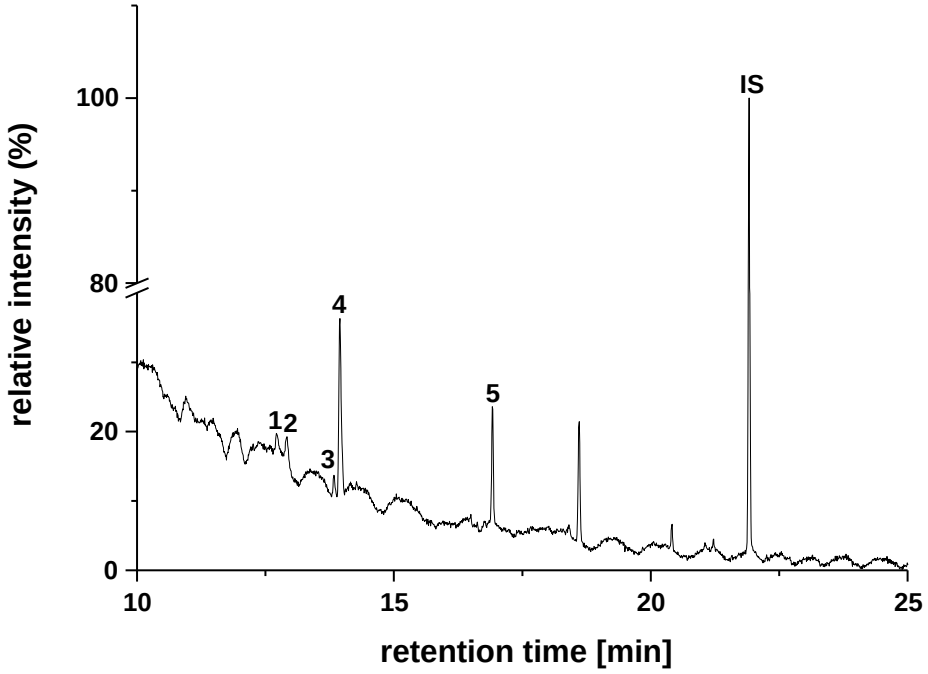
mutant G7Q/M290L, crude extract



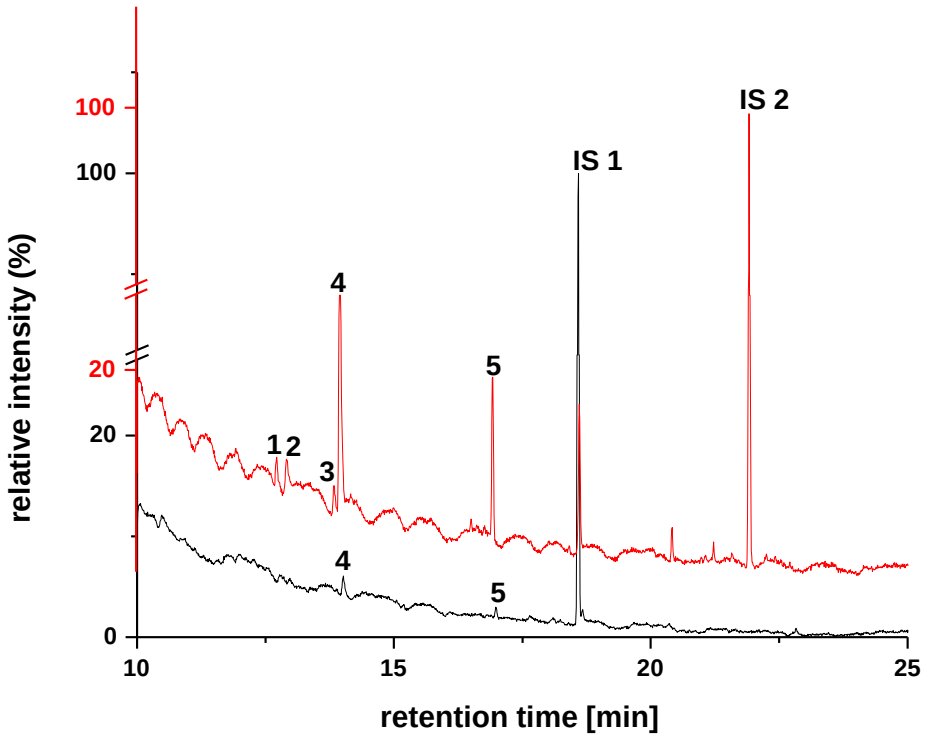
mutant T279A, crude extract



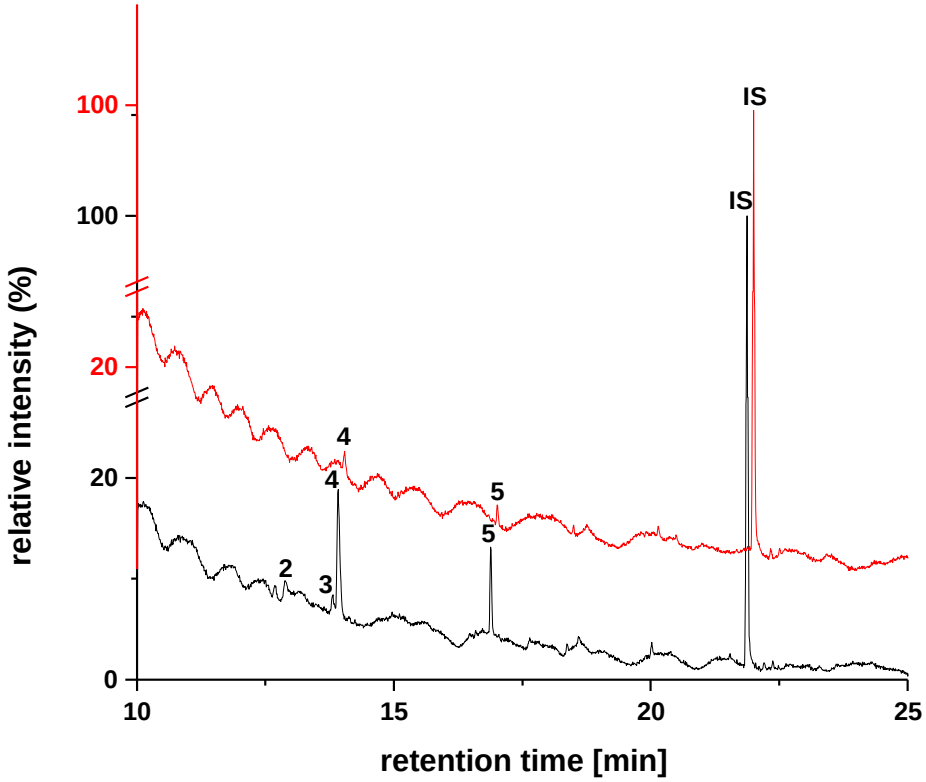
mutant M290L, crude extract



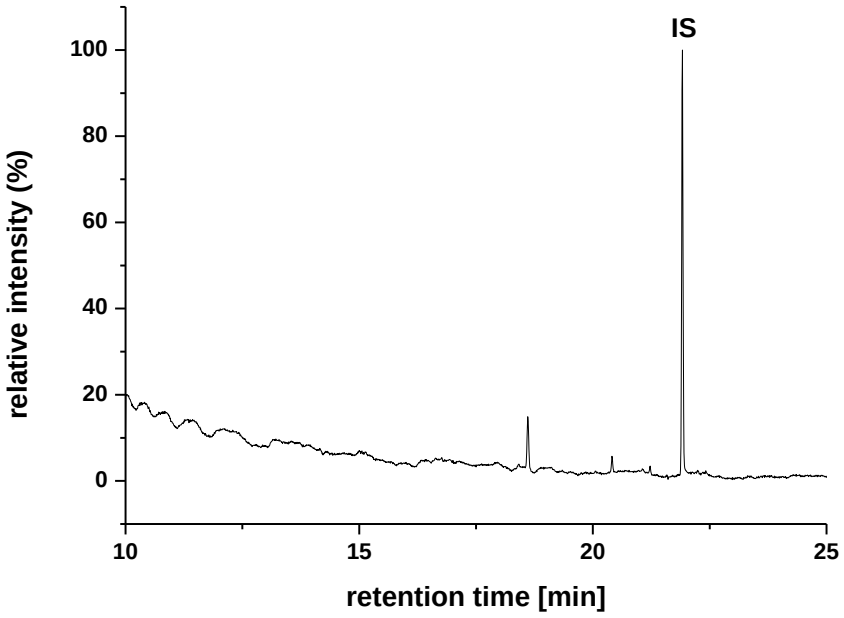
mutant H298D, crude extract



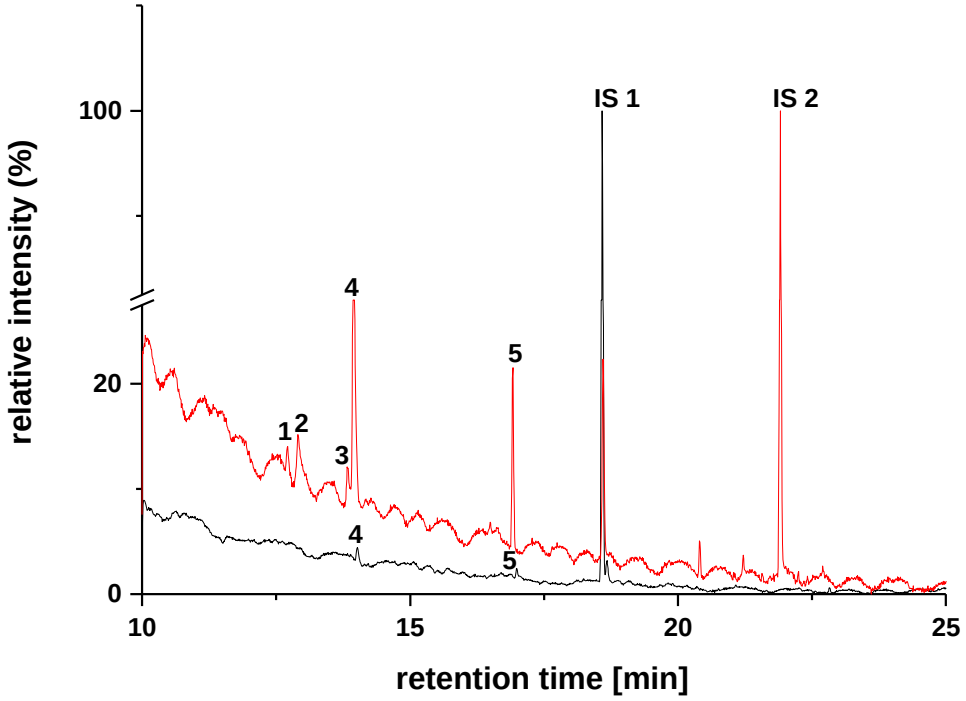
mutant M290L/H298D, crude extract



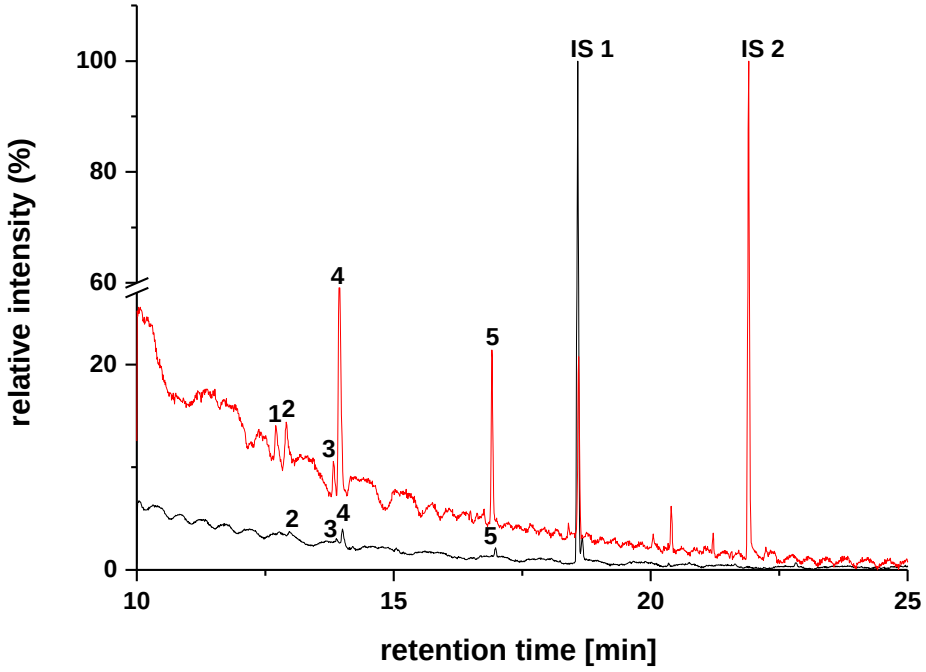
mutant F300I, crude extract



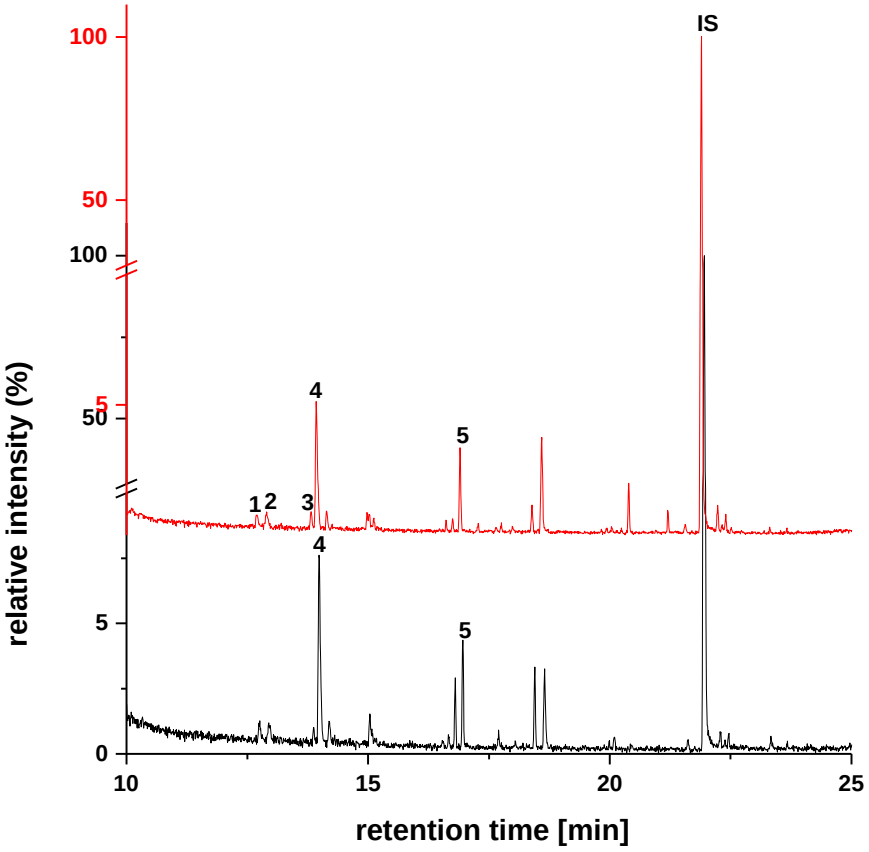
mutant I305T, crude extract



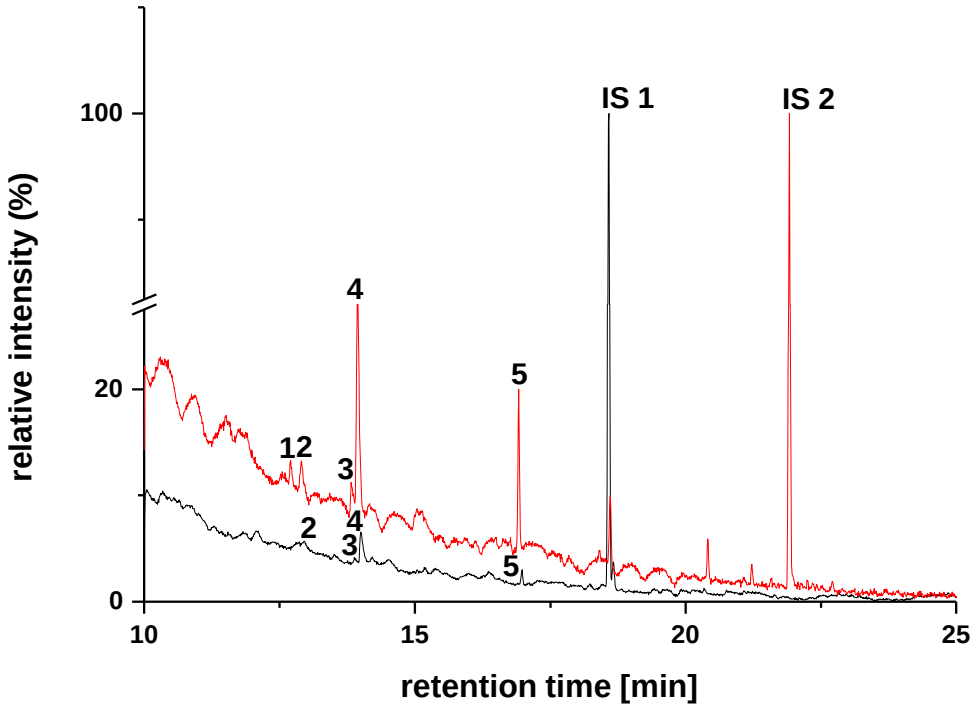
mutant N324D, crude extract



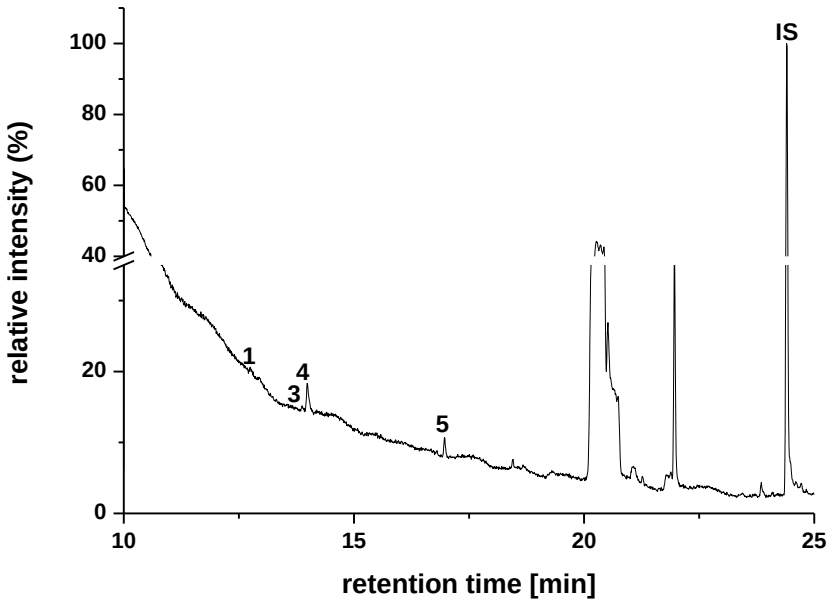
mutant EE335/336-deleted, crude extract



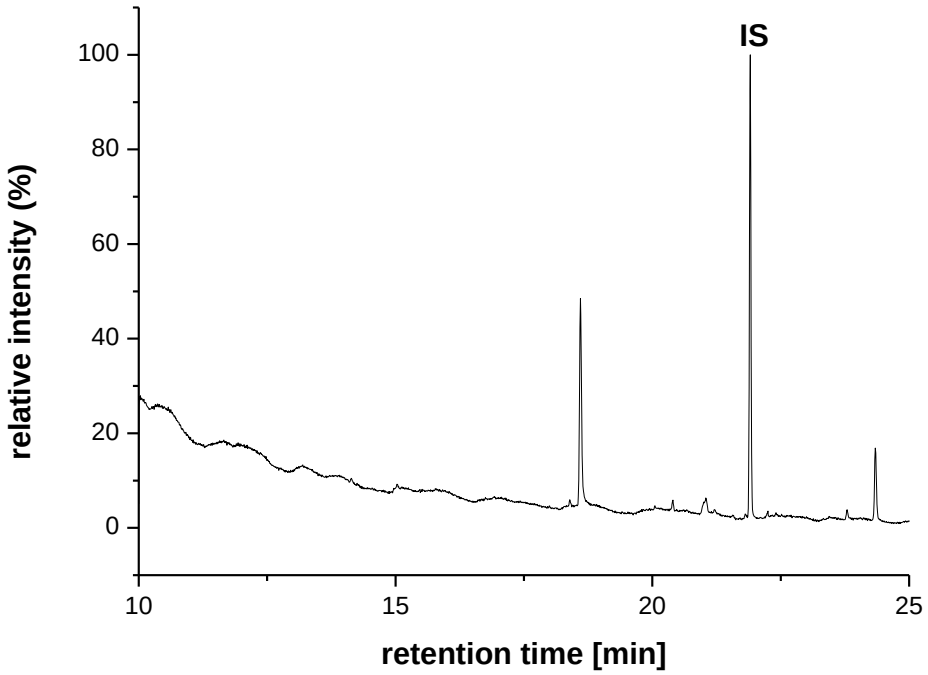
mutant A355S, crude extract



mutant Y446A, crude extract

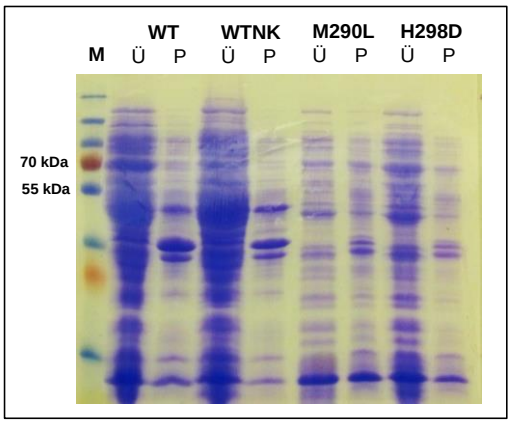


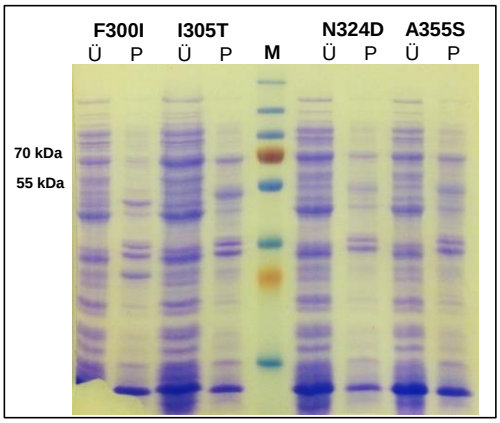
mutant Y502V, crude extract

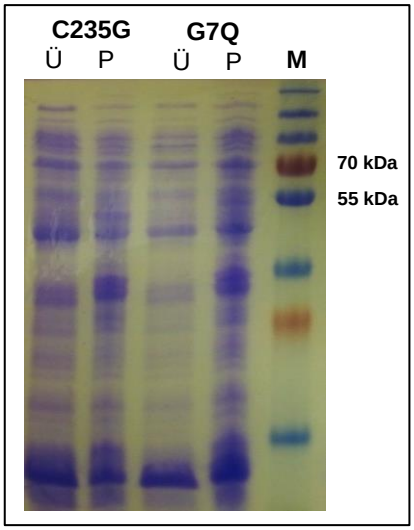


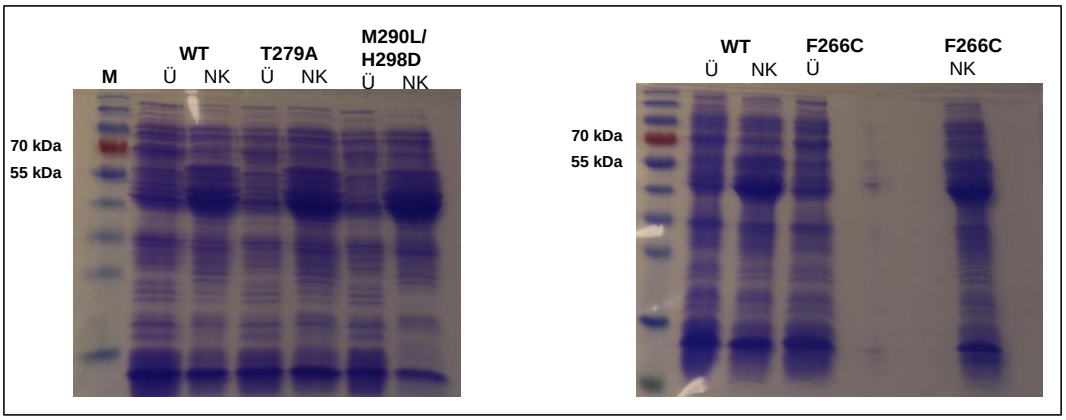
Towards heterologous overexpression of the mutant enzymes in *E. coli* and cell lysis the supernatant (Ü) and the diluted pellet (P) were applied on a SDS gel and stained with Coomassie Brilliant Blue R-250.

The enzyme has a molecular weight of 68 kDa. WT: wild type enzyme, Ü: supernatant (crude extract), P: pellet, M: PageRuler Prestained Protein Ladder, Thermo Fisher Scientific. 18 µl sample + 3 µl 5x loading dye.







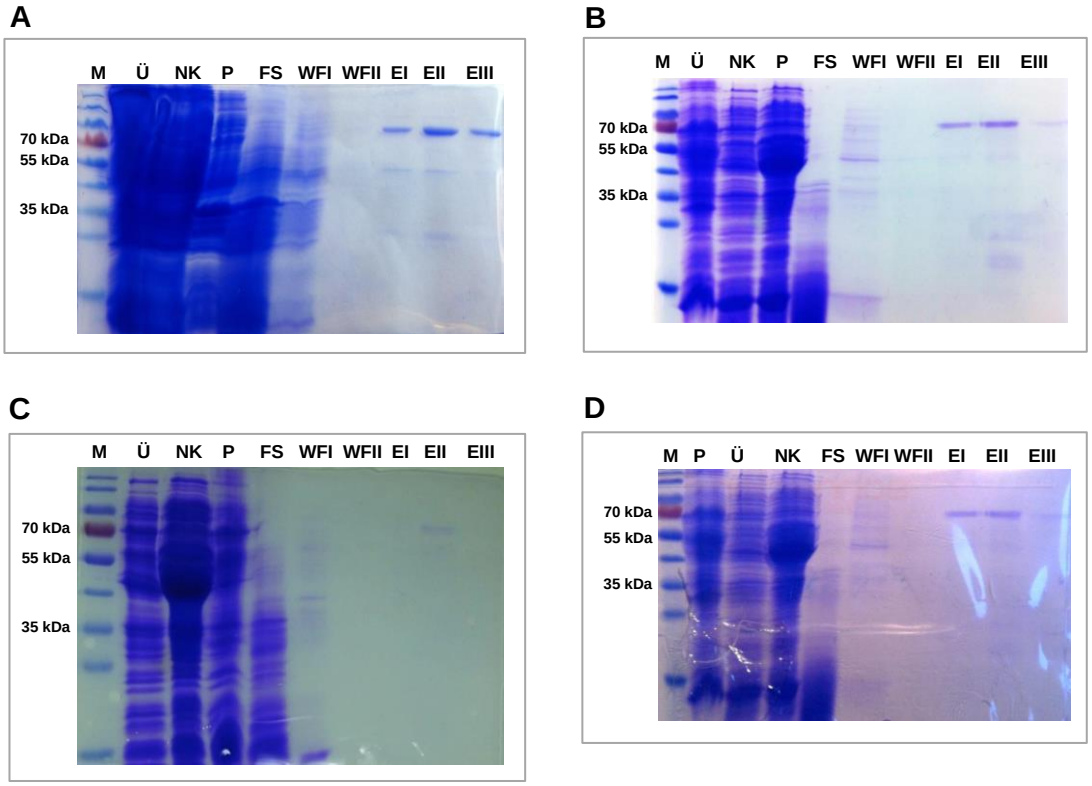


Supplement 5

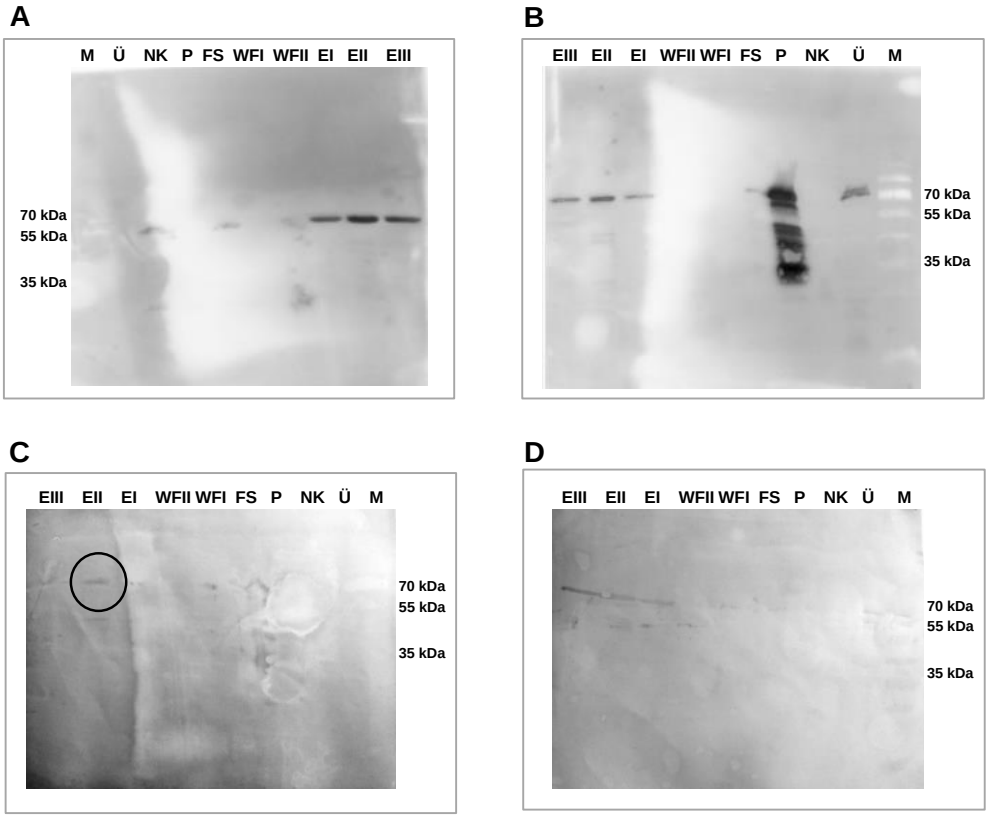
In supplement 5 are shown the SDS-PAGE and Western Blot analysis of the wild type enzyme and the three mutant enzymes (F266S, F266T, F266V). The enzyme has a molecular weight of 68 kDa.

M: PageRuler Prestained Protein Ladder, Thermo Fisher Scientific, Ü: supernatant (crude extract), NK: negative control, P: pellet, FS: flow through, WFI: wash fraction I, WFII: wash fraction II, EI: eluate fraction I, EII: eluate fraction II, EIII: eluate fraction III. **A**: wild type 1,8-cineole synthase from *N. suaveolens*, **B**: mutant enzyme F266S, **C**: mutant enzyme F266T, **D**: mutant enzyme F266V.

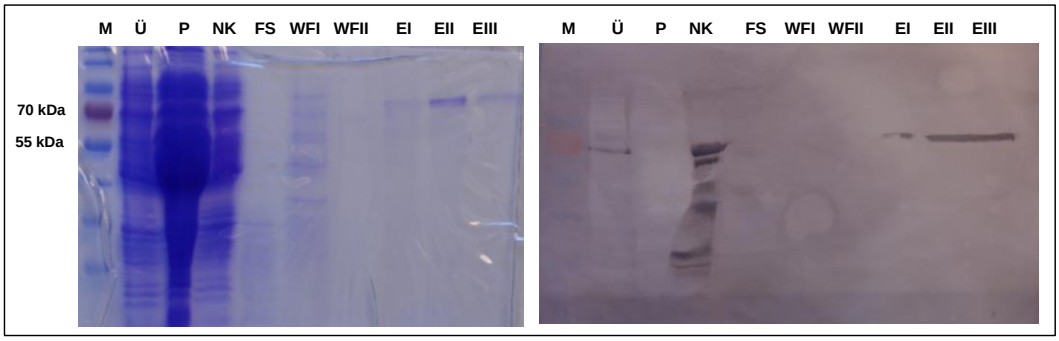
SDS-PAGE



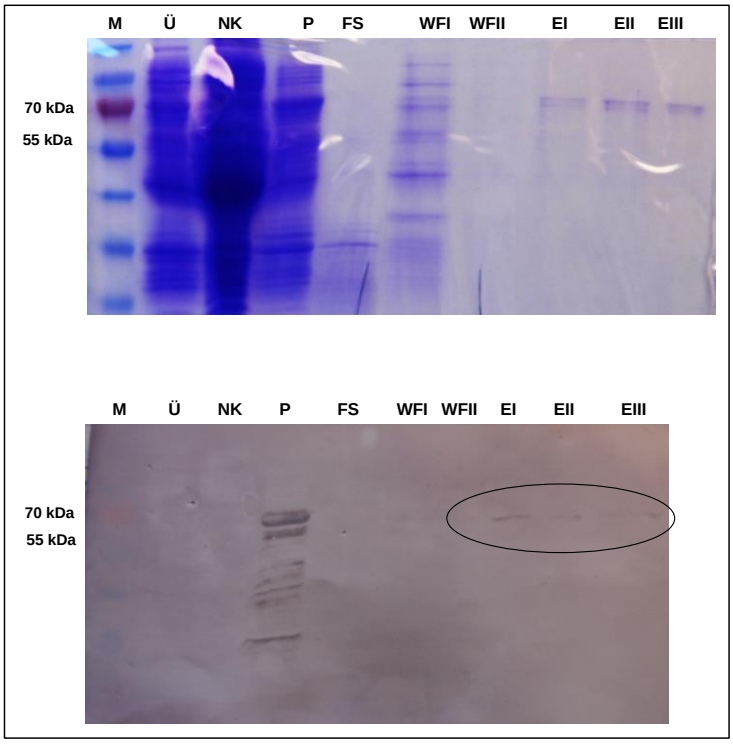
Western Blot



wild type enzyme

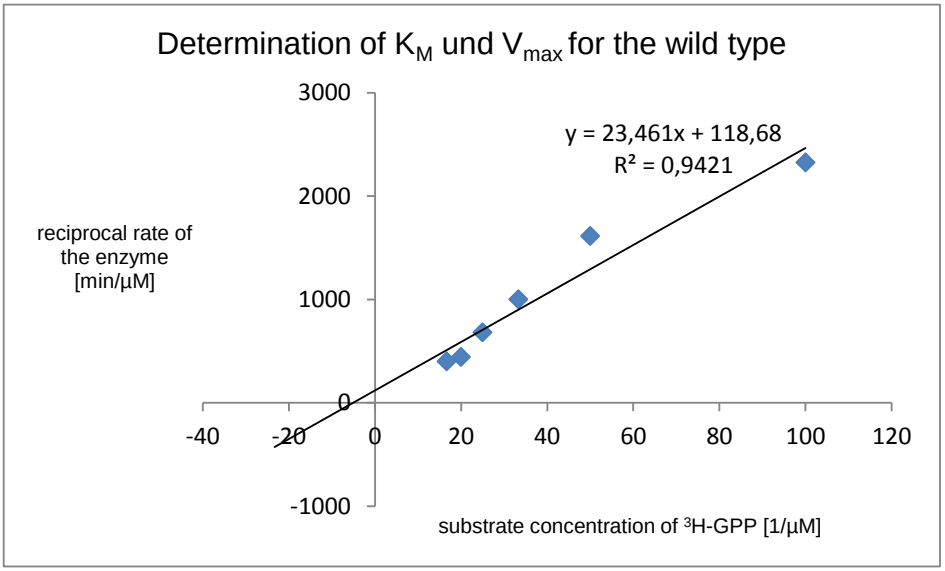


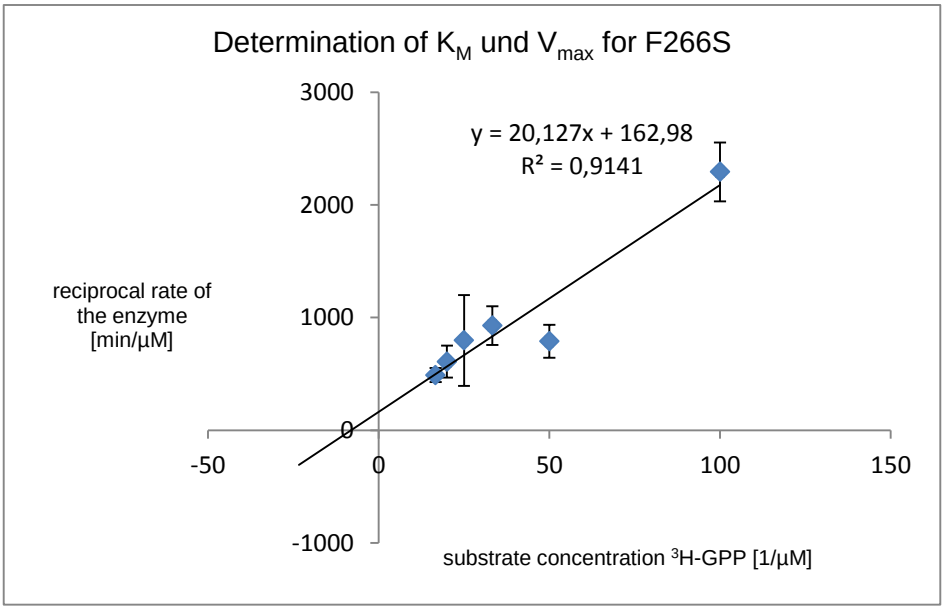
mutant F266S

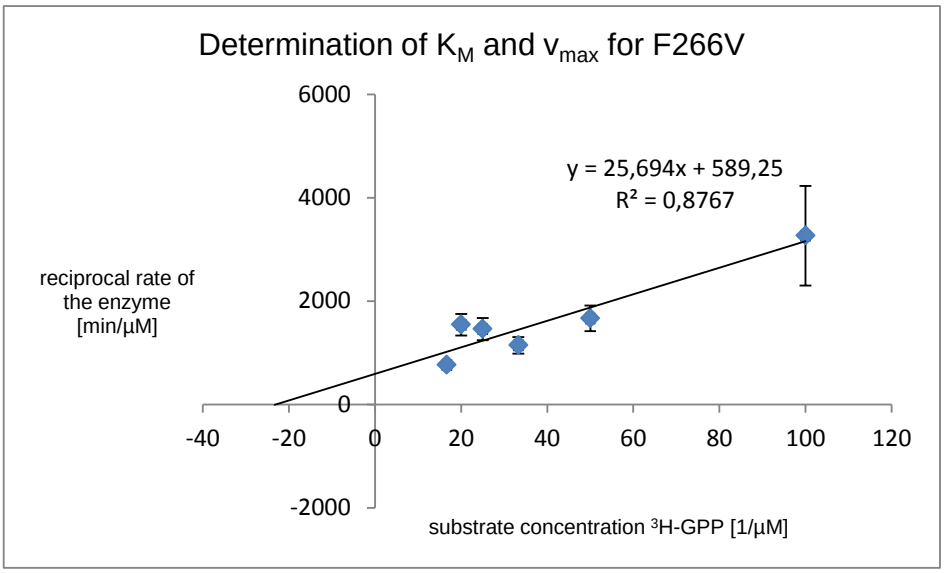


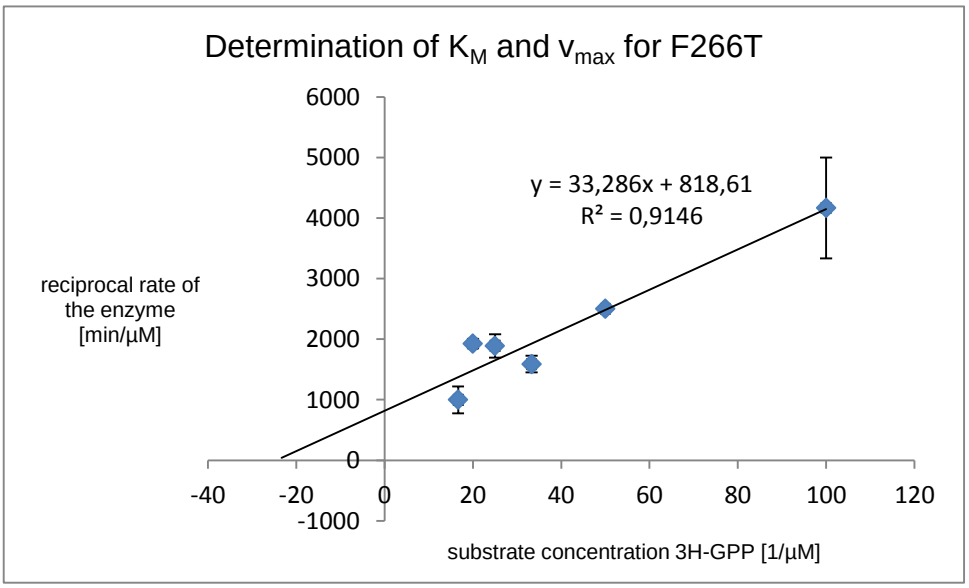
Supplement 6

In supplement 6 are shown the Lineweaver-Burk plots of the purified wild type enzyme and the three purified mutant enzymes (F266S, F266T, F266V).
The determination of K_M and v_{max} was performed with 1 µg enzyme and tritium labelled GPP for 6 min at 41,5 °C.






Determination of K_M and v_{max} for F266V
y = 25,694x + 589,25
R² = 0,8767
reciprocal rate of the enzyme [min/µM]
substrate concentration ³H-GPP [1/µM]
6000
4000
2000
0
-2000
-40
-20
0
20
40
60
80
100
120


Determination of K_M and v_{max} for F266T
y = 33,286x + 818,61
R² = 0,9146
reciprocal rate of the enzyme [min/µM]
substrate concentration 3H-GPP [1/µM]
6000
5000
4000
3000
2000
1000
0
-1000
-40
-20
0
20
40
60
80
100
120

Danksagung

Ich danke Frau Prof. Dr. Birgit Piechulla für die Möglichkeit die vorliegende Arbeit in der Arbeitsgruppe Biochemie anfertigen zu können, sowie für die Beratung.

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Ich gebe folgende Erklärung ab:

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2. Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen habe ich als solche kenntlich gemacht.

Rostock, 20. März 2015

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Anne Brosemann