Acetogene Aromastoffe aus Basidiomyceten

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Zusammenfassung

Aromastoffe spielen eine wichtige Rolle in der modernen Lebensmittelindustrie. Dabei bevorzugen Konsumenten nachhaltige und natürliche Produkte. Diese können biotechnologisch produziert werden. Da das Volatilom von Basidiomyceten vergleichbar zum pflanzlichen ist, besteht ein großes Potential für die Identifizierung neuer, geruchsaktiver Substanzen sowie von Enzymen mit außergewöhnlichen Eigenschaften.

Im Rahmen der vorliegenden Arbeit wurde (5*E*/*Z*,7*E*,9)-Decatrien-2-on als neuer Naturstoff mit einem ananasartigen Aroma im Kulturüberstand des *Fomitopsis betulina* identifiziert. Die olfaktorische Charakterisierung strukturverwandter Verbindungen zeigte, dass 10 Kohlenstoffatome mit einer terminalen Doppelbindung sowie eine „L"-förmige Struktur einen ananasartigen Geruchseindruck hervorrufen. Die Biogenese dieses ungewöhnlichen Methylketons wurde weiterführend charakterisiert. Supplementierungen mit 1/2 -^{13}C-Acetat zeigten einen Aufbau über die Polyketid-Biosynthese. Lactat und Pyruvat wurden als Startermoleküle ausgeschlossen.

Weiterhin konnte am Beispiel der Terpenbiosynthese gezeigt werden, dass Medienadditive die acetogene Biosynthese von Basidiomyceten beeinflussten. *Tyromyces floriformis* wurde als Modellorganismus submers mit unterschiedlichen Additiven kultiviert und *α*-Ylangen als Hauptprodukt quantifiziert. Dabei hatten lipophile Zusätze einen positiven und Polysaccharide einen negativen Effekt auf die Sesquiterpenkonzentration. Die Ergebnisse korrelierten mit der Sesquiterpensynthase-Aktivität. Übertragbarkeitsstudien auf weitere Pilze zeigten, dass, abhängig von der ökologischen Nische des Organismus, der Einfluss der Additive variierte.

Schlagworte: Basidiomyceten, Polyketide, (5*E*/*Z*,7*E*,9)-Decatrien-2-on, Sesquiterpene, *α*-Ylangen

Abstract

Aroma compounds display an important role in modern food production. Consumers prefer sustainable and natural products that may originate from biotechnological processes. Since the volatilome of basidiomycetes shows high similarities to higher plants, these organisms provide a great potential for the identification of novel aroma-active compounds and enzymes with unique properties.

In this work the pineapple like (5*E*/*Z*,7*E*,9)-Decatrien-2-one was identified in the culture supernatant of *Fomitopsis betulina* as new natural compound. Olfactory characterization of the product and its analogues revealed that 10 carbon atoms with a terminal double bond and an “L”-shaped structure were required to evoke the pineapple like odor impression. In following-up work the biogenesis of this unusual methyl ketone was characterized. Media supplementation with 1/2 -^{13}C-acetate proved product formation *via* polyketide biosynthesis. Lactate and pyruvate were excluded as starting molecules.

In addition, the effect of media additives on acetogenic biosynthetic pathways of basidiomycetes was shown using sesquiterpene biosynthesis as an example. The model organism *Tyromyces floriformis* was grown in submerged cultures and *α*-ylangene was quantified as major product. Lipidic additives showed a positive effect on the product concentration, whereas polysaccharides had decreasing effects. Results agreed with sesquiterpene synthase activity results. Transferring the results to other basidiomycota showed that results highly depended on the ecological niche of the fungus.

Keywords: basidiomycetes, polyketides, (5*E*/*Z*,7*E*,9)-Decatrien-2-one, sesquiterpenes, *α*-ylangene

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Abkürzungsverzeichnis

AACT	Acetoacetyl-CoA-Thiolase
ACP	Acyl-Carrier Protein
AEDA	*Aroma extract dilution analysis*
A^3X	3^2-*α*-L-Arabinofuranosyl-xylobiose
$A^{2+3}XX$	*2^3,3^3-Di-α-L-arabinofuranosyl-xylotriose*
BHT	*Butylated hydroxyl toluene*
CMK	Cytidyl-Methyl-Kinase
CMS	Cytidindiphosphat-Methylerythritol-Synthase
CoA	Coenzym A
DCM	*Dichloromethane*
DE	*Diethyl ether*
DH	Dehydratase
DIBAL	*Diisobutyl aluminiumhydride*
DMAP	Dimethylallylpyrophosphat
DMF	*Dimethyl formamide*
DXP	1-Desoxy-D-xylulose-5-phosphat
DXR	1-Desoxy-D-xylulose-5-phosphat-Reductoisomerase
DXS	1-Desoxy-D-xylulose-5-phosphat-Synthase
ER	Enolylreduktase
FAS	Fettsäure-Synthase
FDS	Farnesyldiphosphat-Synthase
FID	*Flame ionization detector*
FPP	Farnesylpyrophosphat
G3P	Glycerinaldehyd-3-phosphat
GC	*Gas chromatography*

GDS	Geranyldiphosphat-Synthase
GOPOD	*Glucose oxidase/ peroxidase*
GPP	Geranylpyrophosphat
HBr	*Hydrogen bromide*
HDS	Hydroxy-Methyl-Butenyl-Diphosphat-Synthase
HMGR	HMG-CoA-Reduktase
HMGS	3-Hydroxy-3-methylglutaryl-CoA-Synthase
IDS	IPP/DMAPP-Synthase
IPI	Isopentylpyrophosphat-Isomerase
IPP	Isopentylpyrophosphat
KR	Ketoreduktase
KS	Ketosynthase
LOD	*Limit of detection*
M/AT	Malonyl/Acetyl-Transferase
MDS	Methyl-Erythritol-Cyclo-Diphosphat-Synthase
MeOH	Methanol
MEP	Methylerythritolphosphat
MGK	Mevalonat-Kinase
MS	*Mass spectrometry*
MUFA	*Monosaturated fatty acids*
MVA	Mevalonat
n.d.	*Not detected*
NMR	*Nuclear magnetic resonance spectroscopy*
ODP	*Olfactory detection port*
p.a.	*Pro analysis*
PE	*Pentene/diethyl ether*

PK	Polyketid
PKS	Polyketid-Synthase
PMD	Phosphomevalonat-Decarboxylase
PMK	Phosphomevalonat-Kinase
PUFA	*Polyunsaturated fatty acids*
RT	*Room temperature*
SBSE	*Stir bar sorptive extraction*
SFA	*Saturated fatty acids*
SPME	S*olid-phase microextraction*
STS	Sesquiterpen-Synthase
TE	Thioesterase
TR	*Traces*
XA^{3}XX	*3^3-α-L-Arabinofuranosyl-xylotetraose*

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1 Einordnung der Forschungsthemen und der wichtigsten Erkenntnisse aus den Publikationen im Kontext der wissenschaftlichen Literatur

1.1 Biotechnologische Aromastoffproduktion

1.1.1 Kommerzielle Bedeutung natürlicher Aromastoffe

Aromastoffe spielen seit Entstehung der Menschheit eine wichtige Rolle in ihrem Alltag. Bis zum Beginn des 19. Jahrhunderts wurden ausschließlich natürliche Aromastoffe in Form von Pflanzenextrakten sowie ätherische Ölen eingesetzt. Anwendungen waren nicht nur spirituelle Riten sowie die tägliche Körperpflege, sondern auch das Balsamieren von Leichen, Infektionsschutz, Wundversorgung sowie der Einsatz zur Schmerzlinderung (Ziegler 2007). Die Verfügbarkeit war jedoch durch ineffiziente Prozesse sowie überwiegend pflanzliches Ausgangsmaterial limitiert, sodass Aromaextrakte vorwiegend wohlhabenden Menschen vorbehalten waren.

Das frühe 19. Jahrhundert gilt als Geburtsstunde der modernen Aromastoffindustrie. Erstmals wurden aromaaktive Verbindungen erkannt, isoliert und wenig später chemisch synthetisiert. Zimtaldehyd war einer der ersten isolierten Aromastoffe (1834 aus Zimt) und wurde nur zehn Jahre später erstmals chemisch synthetisiert (Swift 1999). Revolutioniert wurde die Aromaforschung durch die Entwicklung des Gaschromatographen in den 1950ern (Brenna und Parmeggiani 2017). Dies schuf die Voraussetzung für die Isolierung und Charakterisierung zahlreicher aromaaktiver Substanzen sowie den Grundbaustein moderner Aromaanalytik.

Der weltweite Umsatz der Aromen-Industrie steigt jährlich um 4 bis 5 % (Ben Akacha und Gargouri 2015). 2017 wurden insgesamt 24.8 Mrd. US$ umgesetzt, und bis 2022 soll der Umsatz weiter auf etwa 36 Mrd. US$ steigen (International Flavors & Fragrances Inc. 2018). Heutzutage werden die meisten Aromaformulierungen chemisch synthetisiert oder aus pflanzlichen Quellen extrahiert (Ben Akacha und

Gargouri 2015). Aktuell wird geschätzt, dass weniger als 15 % aller Aromastoffe biotechnologisch produziert werden. Davon entstammen bereits über 5 % der heterologen Produktion (Krammer 2020). Der Anteil sensorisch aktiver Zusatzstoffe aus biotechnologischer Produktion hingegen übersteigt den der Aromastoffe um 20 %, wobei geschätzte 10 % aller biotechnologischen Produkte aus gentechnisch modifizierten Organismen stammen (Krammer 2020).

Chemosynthese von Aromastoffen führt zu hohen Produktmengen, benötigt jedoch meist harte Reaktionsbedingungen sowie organische Lösungsmittel. Entstandene Produkte sind oft Racemate und enthalten neben dem Zielprodukt unerwünschte Nebenprodukte, welche in aufwändigen Reinigungsverfahren abgetrennt werden müssen (Hirschmann *et al.* 2019). So erzeugte Produkte gelten weder als „natürlich“ noch als nachhaltig und werden heutzutage von den Konsumenten nicht bevorzugt.

Die Extraktion aus natürlichen Quellen wie Pflanzen stellt zwar einen einfachen Prozess dar, steht aber in direkter Konkurrenz zur agroindustriellen Nahrungsmittelproduktion. Grund dafür ist, dass einerseits Lebensmittel als Aromastoffquellen dienen und andererseits Ackerflächen für den Anbau nicht verzehrbarer Aromastoffe verwendet werden (Bel-Rhlid *et al.* 2018).

Biotechnologische Prozesse empfehlen sich durch milde Reaktionsbedingungen und Ressourcen schonende Prozesse für eine stereoselektive Produktbildung ohne viele Nebenprodukte. Dies führt zu einer Reduktion von Kosten und Aufwand für Reinigungsprozesse (Lin und Tao 2017). Die biotechnologische Produktion kann sowohl in Form einer enzymatischen Biotransformation als auch im Ganzzellexpressionssystem erfolgen. Ein Vergleich beider Produktionsprozesse zeigt grundlegende Unterschiede. Borup *et al*. verglichen beide Prozesse am Beispiel des

fruchtigen Ethyl-3-hydroxybutyrats, welches in frischer Kiwi, aber auch in Wein und Trauben vorkommt (Borup *et al.* 1981; Jordán *et al.* 2002; Augustyn und Marais 1982). Dabei wurde gezeigt, dass sich die enzymatische Biokatalyse durch eine höhere Produktausbeute und Reinheit hervorhebt (Borup *et al.* 1981). In diesem Beispiel besaß das Ganzzellexpressionssystem eine höhere Gesamteffizienz, da Cofaktoren intrazellulär regeneriert und Enzyme durch das zelluläre Milieu stabilisiert wurden. Weiterhin konnte auf eine kostenintensive Enzymreinigung verzichtet werden (Borup *et al.* 1981). Nichtsdestotrotz können in Zellen unerwünschte Nebenreaktionen ablaufen, welche die Gesamteffizienz des Expressionssystems reduzieren. Dies umschließt die Weiterreaktion des Zielproduktes sowie die Produktion unerwünschter und cytotoxischer Nebenprodukte (Jeromin und Bertau 2005).

Einen klaren Vorteil bietet das Ganzzellexpressionssystem bei der *de-novo*-Biogenese im Rahmen des zellulären Primär- oder Sekundärmetabolismus. Viele biochemische Prozesse laufen mit aktivierter Essigsäure, dem Acetyl-Coenzym A (CoA) ab. Die so genannten Acetoide bilden eine heterogene Naturstoffgruppe (Habermehl *et al.* 2002). Zu ihnen gehören unter anderem Isoprenoide, Lipide sowie Polyketide. Viele aromaaktive Verbindungen werden den Acetoiden zugeordnet.

1.1.2 Industrielle Bedeutung der biotechnologischen Aromastoffproduktion

Heutzutage bieten eine Vielzahl an Unternehmen wie *Symrise*, BASF, *Amyris* oder *Ginkgo Bioworks* biotechnologisch produzierte Aromastoffe an (Krammer 2020). Hierbei erfolgt die Produktion fermentativ und nicht mittels Biotransformation.

Eine Übersicht etablierter Biotransformationsprozesse zeigt Tabelle 1.1.1. Dabei ist auffällig, dass vorwiegend Aromastoffe aus dem Fettsäureabbau über Biotransformationen produziert werden. Beispiel hierfür ist die Produktion von *trans*-2-Hexenal der Firma *Axxence*. Das Produkt wird aus Linolsäure mittels Lipoxygenase,

Hydroperoxid-Lyase und einer Isomerase gebildet (Činčala *et al.* 2015). Eine Ausnahme bietet der einzige bekannte industrielle Prozess mit Basidiomyceten, bei welchem es sich um eine Oxidation von (+)-Valencen zu (+)-Nootkaton durch lysiertes *Pleurotus sapidus* Myzel handelt.

Tabelle 1.1.1. Einige typische industrielle Produkte aus Biotransformationsprozessen (frei nach Klein und Hilmer 2013)

Rohstoff	**Aromastoff**	**Biokatalysator**	**Marktpreis (2013)**
Carbonsäure + Alkohol **Ester + Alkohol** **Ester + Carbonsäure**	Carbonsäureester z. B. 2-Methylbutyrat, Ethylbutyrat	Lipasen z. B. *Candida antarctica*	< 100 €/ kg
Leinsaatöl, Pflanzenöl	*trans*-2-Hexenal, *cis*-3-Hexenol	Lipoxygenase, Hydroperoxid-Lyase	< 1.000 €/ kg
Pflanzenöl	Ethyl-*trans*-2-*cis*-4-decadienoat	Lipasen z. B. *Candida antarctica*	> 1.000 €/ kg
Menthylbenzoat	Menthol	Lipase	< 100 €/ kg
(+)-Valencen	(+)-Nootkaton	*Pleurotus sapidus*	> 1.000 €/ kg

Weiterhin wird eine Vielzahl von Aromastoffen fermentativ produziert (Tabelle 1.1.2). Dabei ist die Produktion natürlichen Vanillins einer der bekanntesten Prozesse. Das Produkt wird bei der submersen Kultivierung von *Amycolatopsis spec.* unter Zugabe der ferulasäurereichen Reiskleie gebildet (Solvay Deutschland 2019; Krammer 2020). Wenige Firmen bieten biotechnologisch produzierte Terpenformulierungen an (Krammer 2020). Eine davon ist *Conagen*, welche natürliche blumige Aromaformulierungen anbietet. Besonders Bakterien, Hefen und Ascomyceten sind als Produktionsorganismen in der Industrie etabliert (Krammer 2020).

Tabelle 1.1.2. Übersicht industrieller Produkte aus Fermentationsprozessen (frei nach Klein und Hilmer 2013)

Rohstoff	Aromastoff	Biokatalysator	Marktpreis (2013)
Ferulasäure (Reiskleie)	Vanillin	*Amycolatopsis* spec.	< 1.000 €/ kg
Alkohole	Carbonsäuren z. B. Propionsäure, Buttersäure	*Gluconobacter* spec. *Acetobacter* spec.	< 100 €/ kg
Ricinolsäure, Rizinusöl	γ-Decalacton	*Yarrowia lipolytica*	< 1.000 €/ kg
Ethylcaprylat	γ-Octalacton	*Mucor circinelloides*	< 1.000 €/ kg
Fettsäuren	Ketone z.B. 2-Pentanon, 2-Nonanon	*Penicillium roqueforti*	< 100 €/ kg
Milch, Molke	Acetoin, Diacetyl	*Streptococcus diacetilactis*, *Streptococcus lactis*	< 100 €/ kg
Phenylalanin	Phenylethanol	*Saccharomyces cerevisiae*	< 1.000 €/ kg
Melasse	Pyrazine	*Saccharomyces cerevisiae*	> 1.000 €/ kg

1.1.3 Basidiomyceten als Produktionsorganismen

Die biotechnologische Aromastoffproduktion mit Basidiomyceten im Ganzzellexpressionssystem ist publiziert, jedoch bisher nicht außerhalb des Labormaßstabs erprobt (Berger und Zorn 2004). Basidiomyceten reagieren stark auf Inhomogenitäten und Verunreinigungen im Kulturmedium. Diese erschweren das Prozessdesign durch hohe Batch zu Batch Variabilität (Lomascolo *et al.* 1999). Zusätzlich wird die Prozesskontrolle durch das filamentöse Wachstum beeinträchtigt, welches die Instrumentierung des Reaktorsystems insbesondere Sonden stört. *Ustilago*, *Pseudozyma*, *Dacryopinax* oder *Moniliella* zeigen in Submerskultur unter extremen Bedingungen hefeähnliches Wachstumsverhalten. Sie werden zwar im

Portfolio von entwickelnden Firmen wie der *PROvendis* GmbH als Expressionsorganismus angeboten, jedoch wenig eingesetzt.

Nichtsdestotrotz bieten Basidiomyceten ein enormes Potential im Rahmen der Aromastoffproduktion. Das breite Produktspektrum erlaubt die Identifizierung von neuen Aromastoffen sowie Enzymen mit einzigartigen Eigenschaften. Gesteigertes Verständnis von Stoffwechselwegen sowie beteiligter Schlüsselenzyme ermöglicht die Übertragung auf heterologe Expressionssysteme. Dabei ähnelt die *Codon Usage* der Basidiomyceten den Hefen oder Ascomyceten. Die heterologe Produktion von Aromastoffen mit *in vitro* Pflanzenzellen ist aufgrund der veränderten *Codon Usage,* des langsamen Wachstums und der Infektionsanfälligkeit deutlich schwieriger.

1.2 Polyketide

1.2.1 Polyketide in Basidiomyceten

Polyketide bilden eine heterogene Gruppe der Naturstoffe, welche besondere Bedeutung als Wirkstoffe erlangt haben (Civjan 2012; Cox 2007; O'Hagan 1991). 2005 wurden mit über 20 zugelassenen Präparaten 0,3 % aller gescreenten Polyketide kommerziell produziert (typischerweise werden 0,001 % aller gescreenten Wirkstoffe zugelassen). Damit ist der prozentuale Anteil industriell nutzbarer Verbindungen 300-mal höher als in anderen Stoffklassen (Weissman und Leadlay 2005).

Polyketide werden kommerziell unter anderem als Antibiotika, Fungizide, Cholesterinsenker, Herzmedikamente, Immunsuppressiva und als Chemotherapeutika eingesetzt (Shen 2003; Civjan 2012; Staunton und Weissman 2001). Weiterhin finden Produkte dieser Stoffklasse Anwendung als Schädlingsbekämpfungsmittel, Toxine, aber auch als natürliche Farbstoffe (Hertweck 2009). Für den Produktionsorganismus wirken die Verbindungen als Pigmente und Virulenzfaktoren

sowie für die inter- und intraspezifischer Kommunikation bis hin zu Verteidigungsmechanismen (Weissman und Leadlay 2005). Es wird davon ausgegangen, dass ihre Rolle komplexer ist als bisher bekannt.

Als Geburtsstunde der Polyketidforschung gilt die Entdeckung des Orcinols 1893 durch James Collie (Collie und Myers 1893). Die Grundlagen der Biogenese wurde jedoch erst 60 Jahre später von Birch während der Arbeiten an pilzlichen Polyketiden aufgeklärt (Birch und Donovan 1953). Trotz der langen Historie wurde erst mit modernen molekularbiologischen Methoden die Polyketid-Biosynthese verstanden. In den 1980er Jahren identifizierten Hopwood *et al*. die ersten Gene und charakterisierten entsprechende Gencluster (Malpartida und Hopwood 1984; Hopwood 1997). Zehn Jahre später eröffneten Beck *et al*. ein neues Wissenschaftsfeld mit der Charakterisierung des 6-Methylsalicylsäure-Synthase Gen-Clusters aus *Penicillium patulanum* (Beck *et al.* 1990). Die Polyketid-Biosynthese ist heutzutage ein wichtiges Forschungsfeld. Dies beinhaltet die Identifizierung neuer bioaktiver Moleküle von kommerziellem Wert sowie die Manipulation produzierender Enzymkomplexe durch gentechnische Methoden, um das Produktspektrum weiter zu verbreitern (Shen 2003; Horinouchi 2008; Zhang und Tang 2008).

Polyketide aus Pilzen zeigen eine besonders hohe strukturelle Variabilität. Sowohl einfache monocyclische Verbindungen wie Tetraketide z. B. 6-Methylsalicylsäure als auch komplexe polyzyklische Aromaten sind bekannt (Cox und Simpson 2009). Pilzliche Polyketide sind vor allem Tetra- (C_8) oder Decaketide (C_{20}) (Tkacz und Lange 2004).

1.2.2 Polyketid-Biosynthese

Trotz struktureller Inhomogenität vereint alle Polyketide ein grundlegender Mechanismus der Biosynthese. Die Nähe zur Fettsäuresynthese wurde bereits 1953

von Birch und Donovan beschrieben (Birch und Donovan 1953). Molekularbiologische Techniken zeigten 2007 auf genomischer Ebene hohe Homologien in den katalytischen Zentren der Polyketid- (PKS) und Fettsäure-Synthase (FAS). Beide Enzyme eint nicht nur der identische Mechanismus zur Kettenverlängerung, sie verwenden auch dieselben Bausteine. In beiden Biosynthesen wird über eine *Claisen*-Kondensation die C-C-Bindung zwischen einem aktivierten Startermolekül und einem Thioester-CoA geknüpft und so ein linearer Polyketoester synthetisiert.

In jedem Schritt der Kettenverlängerung wird ein Startermolekül, typischerweise die Acetyleinheit des Acetyl-Coenzym A (Acetyl-CoA), vom Acyl-Carrier-Protein (ACP) erkannt und über einen Cysteinrest auf die Ketosynthase (KS) übertragen (Abbildung 1.2.1). Der Baustein zur Kettenverlängerung, meist Malonyl-CoA, wird anschließend am ACP gebunden. Der Carbonylkohlenstoff der Acetyleinheit wird nukleophil von einem Malonat angegriffen. Diese C-C-Knüpfung findet unter Decarboxylierung des Malonats statt. Als Produkt entsteht eine β-Ketoacylkette, welche wiederum am ACP gebunden ist. Die Ketogruppen werden abhängig vom Zielprodukt enzymatisch einfach oder mehrfach reduziert. Die Ketoreduktase (KR) reduziert die entsprechende Ketogruppe zur Hydroxygruppe, welche über die Dehydratase (DH) entfernt wird. Das Produkt kann über eine Enolylreduktase (ER) weiter reduziert werden. Für eine weitere Kettenverlängerung wird die Kette analog zum Startermolekül auf die KS übertragen. Die Thioesterase (TE) katalysiert die Abspaltung des CoAs. Anschließend liegt die Kette als freie Säure oder als Acylester vor (Staunton und Weissman 2001).

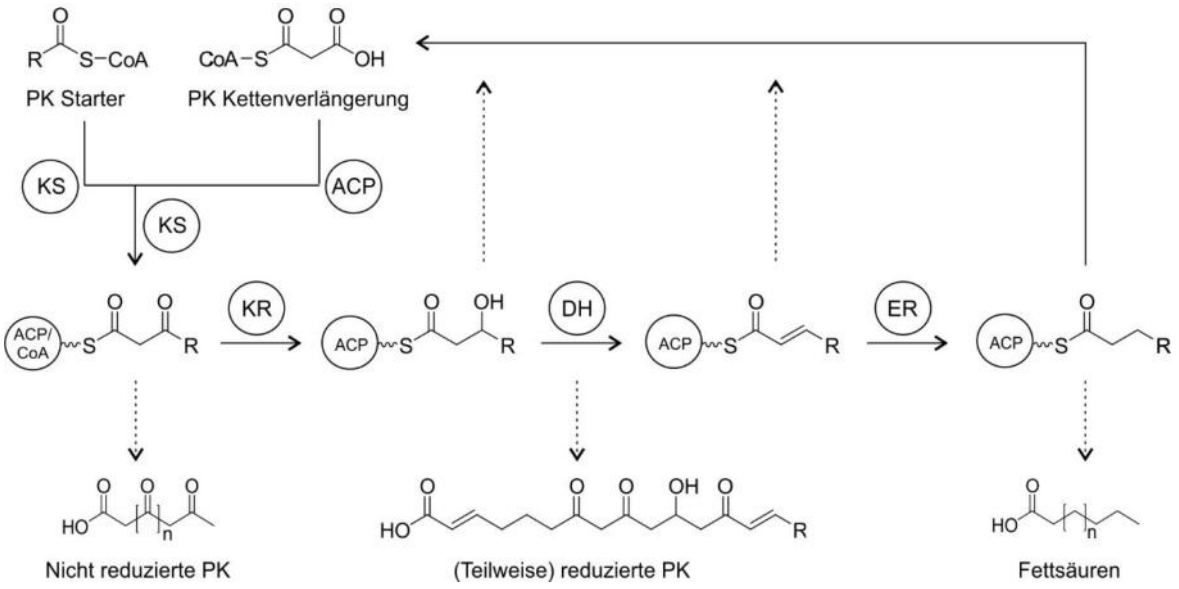


Abbildung 1.2.1. Schematische Darstellung der Kettenverlängerung der Polyketid- (PK) Biosynthese. Bausteine liegen in der aktivierten Form als Coenzym A (CoA) vor. Beteiligte Enzyme sind die Ketosyntase (KS), das Acyl-Carrier-Protein (ACP), die Ketoreduktase (KR), die Dehydratase (DH) sowie die Enolylreduktase (ER) (frei nach Weissman 2009).

Zentraler Unterschied der Fettsäure- und Polyketid-Biosynthese ist, dass FAS ausschließlich die Anzahl der Kettenverlängerungen regulieren, wohingegen PKS die Wahl der Startermoleküle sowie den Grad der Reduktion und Dehydratisierung während der Kondensationsreaktion bestimmen (Cox und Simpson 2009). Weiterhin besitzen PKS eine Malonyl/Acetyl-Transferase (M/AT), welche Bausteine erkennt und auf das ACP überträgt (Staunton und Weissman 2001).

Charakteristisch für die Polyketid-Biosynthese ist die anschließende Prozessierung der Kette. Die bisher linearen Ketten können alkyliert werden oder *via* Aldol-Reaktion, *Claisen*-Kondensation sowie *Diels-Alder*-Reaktionen cyclisieren (Schrader und Bohlmann 2015). Die entstandene so genannte primäre Kernstruktur wird isomerisiert und erneut cyclisiert. Die entstandene zweite Kernstruktur wird final modifiziert über Oxygenierungen, Halogenierungen, erneute Alkylierungen sowie Glycosylierungen und Methylierungen. Ausprägung und Art der Prozessierung sind genetisch reguliert

und über einen Multienzymkomplex, in welchem die Biosynthese abläuft, gesteuert. Neben cyclischen Strukturen sind auch lineare offene Ketten bekannt, welche oft über Etherbindungen cyclisieren (Nuhn und Wessjohann 2006). Das fertige Polyketid wird von der PKS frei gesetzt.

1.2.3 Enzymologie der Polyketidbiosynthese

Alle PKS sind Multienzymkomplexe mit Ketosynthase-, Acyl-Carrier-Protein- sowie Acyltransferase-Aktivitäten. Die Ausstattung mit Ketoreduktase, Dehydratase, Enolyreduktase sowie Thioesterase ist PKS spezifisch, und Änderungen in Ausstattung und Anordnung beeinflussen das finale Produkt grundlegend. Die multifunktionalen Proteinkomplexe, welche typischerweise zwischen 180 und 250 kDa groß sind, arbeiten iterativ in einem hoch regulierten, komplexen Prozess (Cox und Simpson 2009).

Die Enzymkomplexe werden abhängig von ihren Eigenschaften und Sequenzhomologien klassifiziert. Dies wird noch kontrovers diskutiert. So warnten Müller und Shen bereits in den frühen 2000ern vor der starren Klassifizierung dieser Enzyme in wenige Klassen (Müller 2004; Shen 2003). Das System wurde stetig weiter entwickelt und von ursprünglich drei Klassen auf die in Tabelle 1.2.1 aufgeführten fünf erweitert. Zusätzlich sind Mischformen und Hybride unterschiedlicher PKS-Klassen bekannt (Hertweck 2009).

Die meisten basidiomycetischen PKS werden als iterative Typ I PKS klassifiziert (Hertweck 2009). Dies sind multifunktionelle Enzyme. Dabei wird das Substrat nach der Kettenverlängerung immer wieder auf dieselbe KS übertragen und die Bausteine vom selben ACP erkannt und gebunden. Bakterielle PKS des Typ I hingegen arbeiten nicht iterativ, sondern modular (Shen 2003; Hertweck 2009). Für jeden Schritt der Kettenverlängerung besitzt die PKS eine KS sowie ein ACP. Die Polyketidkette wird

nach jeder Kettenverlängerung auf das nachfolgende Modul übertragen. Ein Produkt bindet niemals zweimal an derselben KS Region. Die Mehrheit der Produkte sind reduzierte Polyketide, welche den Macroliden, Polyethern oder Polyenen zugeordnet werden.

Tabelle 1.2.1. Klassifizierung der PKS (frei nach Weissman 2009; Hertweck 2009)

PKS Typ	Modus	Substrat-aktivierung	Kettenverlängerungs-bausteine	Organismus
Typ I	Modular	ACP	Unterschiedliche	Bakterien, (Protisten)
Typ I	Iterativ	ACP	Malonyl-CoA	Pilze, wenige Bakterien
Typ II	Iterativ	ACP	Malonyl-CoA	Bakterien
Typ III	Iterativ	CoA	Malonyl-CoA	Pflanzen, wenige Bakterien, wenige Pilze
PKS-NRPS Hybrid	Iterativ	ACP	Malonyl-CoA, Aminosäuren	Pilze, Bakterien

Typ III PKS wurden erstmalig 2005 in Pilzen identifiziert (Seshime *et al.* 2005). Dabei handelt es sich um homodimere Enzyme, welche ACP-unabhängig eine iterative Kettenverlängerung durchführen. Typische Produkte sind aromatische Polyketide, zu denen die Flavonoide, Isoflavonoide sowie Anthocyanine zählen (Seshime *et al.* 2005).

Weiterhin sind Hybride aus Typ I PKS und nichtribosomalen Peptid-Synthetasen (PKS-NRPS Hybrid) aus Pilzen bekannt (Hertweck 2009). Resultierende Metabolite sind Polyketid-Peptid-Hybride wie die Fusarine, welche eine Gruppe von Mycotoxinen darstellen (Brown *et al.* 2012).

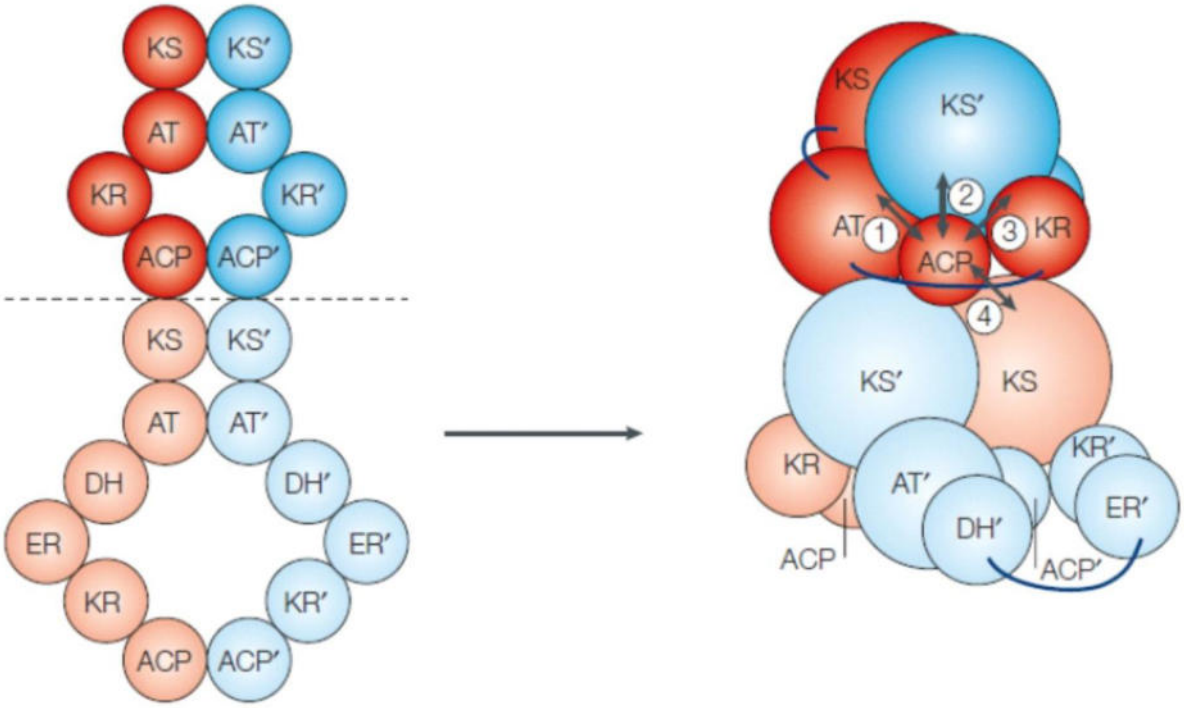


Abbildung 1.2.2. Schematische Darstellung eines PKS Enzymkomplexes. Die Sekundärstruktur der einzelnen Untereinheiten wird so gefaltet, dass nacheinander arbeitende katalytische Zentren in räumlicher Nähe stehen, um die Wege der Kette im Multienzymkomplex zu verkürzen. Ketosynthase (KS), Acyltransferase (AT), Ketoreductase (KR), Acyl-Carrier Protein (ACP), Dehydratase (DH) sowie Enolylreduktase (ER) kommen zusammen, um das Polyketid zu synthetisieren (frei nach Weissman und Leadlay 2005).

Die räumliche Struktur von PKS ist auf Grund ihrer Größe und Komplexität schwer zu analysieren und zu rekonstruieren. Die Erklärung zwischen räumlicher Struktur und Aktivität wird mit dem doppelhelikalen Modell (Abbildung 1.2.2) angestrebt. PKS können aus bis zu acht Untereinheiten bestehen (Oliynyk *et al.* 2003). Dabei weist jede Untereinheit eine homodimere Struktur auf. Homodimere werden so ineinander verdrillt, dass die ACP- und KS-Regionen in räumlicher Nähe zueinander sind (Staunton *et al.* 1996).

Das ACP besitzt eine zentrale Rolle im Multienzymkomplex. Umliegende Regionen kommunizieren mit dem ACP und werden von diesem im Verlauf der Biosynthese koordiniert. Dadurch wird vor allem die Kettenverlängerung beeinflusst. Neben der

Tertiärstruktur prägt die Quartärstruktur das finale Polyketid grundlegend. Dabei sind heutzutage noch nicht alle Mechanismen verstanden (Weissman und Leadlay 2005).

1.2.4 Klassifizierung von Polyketiden

Um die Vielzahl an Polyketiden besser zu unterscheiden, werden sie klassifiziert. Dafür gibt es unterschiedliche Möglichkeiten. Eine wenig verbreitete Methode ist die Klassifizierung abhängig von der Anzahl an Acetatbausteinen. Dabei werden erst Produkte, welche aus mehr als zehn Acetatbausteinen aufgebaut sind, als Polyketid bezeichnet (Nuhn und Wessjohann 2006). Die meisten Pentaketide in Pilzen, zu welchen das in dieser Arbeit vorgestellte (5*E/Z*,7*E*,9)-Decatrien-2-on zählt, sind Naphthalen- sowie Naphthochinon-Derivate. Weiter verbreitet ist die Klassifizierung von Polyketiden in Abhängigkeit von ihrer Struktur. Dabei werden typischerweise auch Decaketide und kleinere Verbindungen berücksichtigt.

Grundlegend werden aromatische und nicht aromatische Polyketide unterschieden. Nicht aromatische Verbindungen werden wiederum abhängig von ihren Charakteristika in Makrolide, Polyether, Endiine sowie Polyene unterteilt (Leeper und Vederas 2000; Hertweck 2009). Trotz der klaren Abgrenzung der Definitionen können nicht alle Produkte eindeutig einer Klasse zugeordnet werden (Weissman und Leadlay 2005).

Zu den aromatischen Polyketiden zählen unter anderem Antibiotika wie die Tetracycline (Leeper und Vederas 2000). Die Strukturen entstehen direkt durch Cyclokondensation der Poly-β-ketointermediate oder der partiell reduzierten Polyketidkette. In Pilzen werden die Cyclisierungen, ähnlich wie die Proteinfaltung durch Chaperone, in speziellen Produkt-Templat-Domänen katalysiert. Dort wächst die Polyketidkette während der Bildung hinein (Crawford *et al.* 2008).

Lineare Polyene, dem auch (5*E/Z*,7*E*,9)-Decatrien-2-on zugeordnet werden kann, sind typische Produkte der durch die PKS katalysierten Kettenverlängerung. Normalerweise werden diese Intermediate im Multienzymkomplex weiter prozessiert, um eine der anderen Produktklassen zu bilden. Nichtdestotrotz sind Produkte bekannt, welche den Polyenen zugeordnet werden. Diese sind meist Pigmente, zu denen unter anderem Piptoporinsäure, Laetiporinsäure oder Melanocrocin zählen.

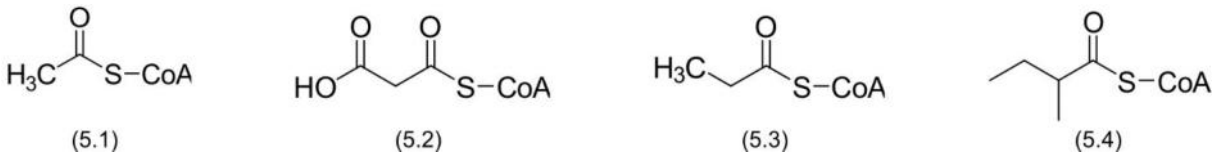
1.2.5 Flüchtige Polyketide

Neben den bekannten wenig flüchtigen Pharmazeutika und Mycotoxinen wurden in den vergangenen Jahren kleinere, flüchtige Polyketide identifiziert (Dickschat 2017). Dabei handelt es sich um lineare Polyene. Das flüchtige 2-Hexen-1-ol ist ein Intermediat der Fusarinsäure, welches vom Polyketidsynthase-Multienzymkomplex (PKS) freigesetzt werden kann (Studt *et al.* 2016). Dickschat schlussfolgerte bei der Arbeit an *Fusarium fujikuroi*, dass ACP Domänen einiger PKS die Kette an unterschiedlichen Punkten während der Biosynthese freisetzen können. Dies resultiert in einem Produktspektrum der PKS, welches neben dem finalen Produkt einzelne Intermediate beinhaltet. Durch spontane Decarboxylierung der β-Ketosäure-Intermediate werden Ketone gebildet, welche im Volatilom identifiziert werden können (Dickschat 2017).

1.2.6 Startermoleküle der Polyketid-Biosynthese

Wie bereits in den vorherigen Abschnitten detailliert erläutert, ist die Kombination von Acetat und Malonat als Starter- und Kettenverlängerungseinheit am weitesten verbreitet. Dabei wird das Acetat, wie in Abbildung 1.2.2 dargestellt, zum Thioester (**5.1**) aktiviert, über das ACP erkannt und auf die KS übertragen. Zusätzlich sind zahlreiche weitere Startereinheiten sowie unterschiedliche Mechanismen der Bindung bekannt (Hertweck *et al.* 2001). Die räumliche Anordnung des Bindungstunnels der KS

für das Coenzym A spielt eine grundlegende Rolle bei der selektiven Bindung der Startereinheit (Ferrer *et al.* 1999; Jez *et al.* 2002). Ortsgerichtete Mutationen führten zu einem breiteren Substratspektrum (Watanabe *et al.* 2007). Wird beispielsweise das Cystein im aktiven Zentrum der KS durch Glutamin ersetzt, wird Malonyl-CoA (**5.2**) anstelle von Acetyl-CoA als Startereinheit erkannt (Bisang *et al.* 1999). Die Bindung erfolgt unter Decarboxylierung, sodass anschließend analog zum Acetyl-CoA eine C2-Einheit an die KS übertragen wird.



(5.1) (5.2) (5.3) (5.4)

Abbildung 1.2.3. Ungewöhnliche Polyketid-Startermoleküle. Übersicht über unterschiedliche Thioester Acetyl-CoA (**5.1**), Malonyl-CoA (**5.2**), Propionyl-CoA (**5.3**) und Isovaleryl-CoA (**5.4**), welche als Polyketid Startereinheiten bekannt sind.

Neben Acetat sind auch andere aktivierte kurzkettige Fettsäuren wie Butyryl-CoA oder Propionyl-CoA als Startermoleküle bekannt (siehe Abbildung 1.2.3). Besonders weit verbreitet ist die Kombination aus Propionat als Starter und Methylmalonat als Molekül zur Kettenverlängerung (Habermehl *et al.* 2002). Propionyl-CoA (**5.3**) wird von der N-terminalen Ladedidomäne der Acyltransferase (AT) erkannt und auf das ACP übertragen. Über *Claisen*-Kondensation dieses Startermoleküls mit Methylmalonat werden unter anderem Makrolide wie das Erythromycin gebildet (Habermehl *et al.* 2002). Isovaleryl-CoA (**5.4**) dient als Startermolekül in der Avermectin-Biosynthese, welches als Antiparasitikum eingesetzt wird (Ikeda *et al.* 1999). Neben linearen Startereinheiten sind zahlreiche cyclische Startermoleküle wie Cyclohexanoyl-CoA sowie phenolische Verbindungen wie das Benzoyl-CoA bekannt (Palaniappan *et al.* 2003; He und Hertweck 2004).

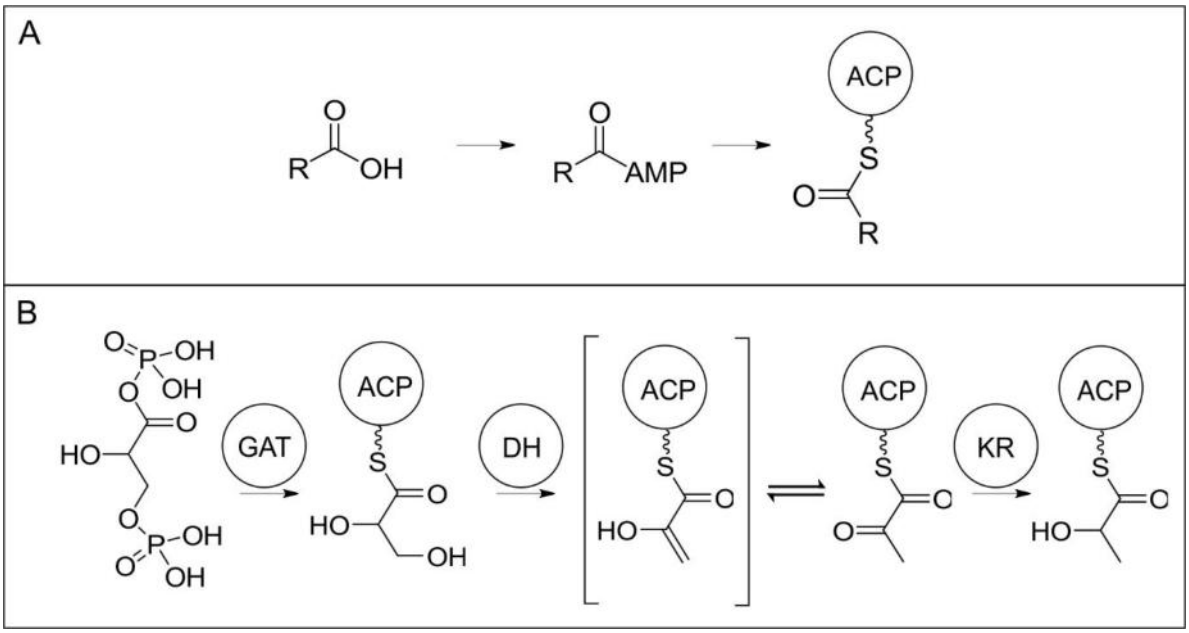


Abbildung 1.2.4. Mechanismus der Bindung nicht aktivierter Startermoleküle. Säuren werden über Adenylierung und Thiolierung an das ACP übertragen. (A) Neben Säuren werden auch phosphorylierte Verbindungen über die GAT an das ACP übertragen. (B) Das gebundene Startermolekül wird vor der Kettenverlängerung entsprechend den Anforderungen des Zielprodukts enzymatisch reduziert. Acyl Carrier Protein (ACP), Glyceryltransferase/ -phosphatase (GAT), Dehydratase (DH), Ketoreduktase (KR). (frei nach He *et al.* 2014; Hertweck 2009).

Wie in Abbildung 1.2.4 (A) dargestellt, binden nicht nur aktivierte Verbindungen, sondern auch freie Säuren an das ACP. Dies ist mit PKS-NRPS Hybriden möglich. Dabei wird die Säure durch Adenylierung der NRPS-ähnlichen Regionen aktiviert. Diese wird thioliert und auf das ACP übertragen (Lowden *et al.* 2001). Ähnliche Bindungsmechanismen sind auch bei phosphorylierten Startermolekülen bekannt (Abbildung 1.2.4 (B)). Diese werden von der Glyceryltransferase/-phosphatase (GAT) erkannt und an das ACP übertragen. Nach der Bindung können DH- und KR-Regionen das Startermolekül weiter reduzieren (He *et al.* 2014).

1.3 Sesquiterpene

1.3.1 Bedeutung von Sesquiterpenen bei den Terpenoiden

Terpenoide bilden mit über 40.000 bekannten Verbindungen die größte Gruppe der Naturstoffe (Schrader und Bohlmann 2015). Sie werden sowohl von Tieren als auch von Pflanzen, Prokaryoten und Pilzen gebildet, wobei pflanzliche Quellen die größte Gruppe darstellen. Aber auch Pilze, insbesondere Basidiomyceten, synthetisieren ein breites Portfolio an Produkten (Kramer und Abraham 2012).

Terpene sind Kohlenwasserstoffe und formal auf kondensierte Isoprenuntereinheiten zurückzuführen. Obwohl Isopren bereits vor über 100 Jahren als biologischer Präkursor ausgeschlossen wurde, basiert die 1987 entwickelte Klassifizierung auf diesem Baustein (Buchanan *et al.* 2015; Wallach 1887). Heutzutage wird zwischen Hemi- (C_5; eine Untereinheit), Mono- (C_{10}; zwei Untereinheiten), Sesqui- (C_{15}; drei Untereinheiten), Di- (C_{20}; vier Untereinheiten), Sester- (C_{25}; fünf Untereinheiten), Tri- (C_{30}; sechs Untereinheiten), Tetraterpenen (C_{40}; acht Untereinheiten) und Polyisoprenen (C_n; > acht Untereinheiten) unterschieden. Cyclisierungen sowie funktionelle Gruppen erhöhen die Diversität der möglichen Verbindungen. Bekannt sind Aldehyd-, Alhohol-, Carbonsäure-, Ester-, Ether-, Glycosid- und Keton-Terpene.

Unter den Terpenen bilden die Sesquiterpene mit über 300 bekannten Grundgerüsten die größte Gruppe an flüchtigen Verbindungen (Abraham und Berger 1994). Erstmals wurden diese 1963 in Pilzen beschrieben (Sprecher 1963). Während die Kohlenwasserstoffe oft einen unspektakulären Geruch haben, finden oxofunktionalisierte Terpenoide oft Einsatz als Aromastoffe (Fraatz *et al.* 2009).

1.3.2 Sesquiterpen-Biosynthese

Zwei unterschiedliche Wege der Terpenbiosynthesen sind bekannt (Abbildung 1.3.1). Der so genannte Mevalonat-Weg (MVA-Weg) ist besonders in Eukaryoten und

Archaeen weit verbreitet, wohingegen in Bakterien die Bausteine über den Methylerythritolphosphat-Weg (MEP-Weg) gebildet werden. Genomanalysen weisen darauf hin, dass einige Pilze neben dem MVA-Weg auch Enzyme für die MEP-Biosynthese besitzen (Lee *et al.* 2016).

Namensgebend für den in Abbildung 1.3.1 (links) dargestellten MVA-Weg ist die Mevalonsäure. Diese entsteht durch Reduktion des (S)-3-Hydroxy-3-methylglutaryl-CoA durch die HMG-CoA Reduktase (HMGR), welches vorher *de-novo* aus Acetyl-CoA gebildet wurde. Die Mevalonsäure wird zweifach phosphoryliert. Das dabei entstandene Pyrophosphat wird decarboxyliert und es entsteht das aktivierte Isopren, das Isopentylpyrophosphat (IPP). Die Isopentylpyrophosphat-Isomerase (IDI) isomerisiert IPP zu Dimethylallylpyrophosphat (DMAP). Zusammen bilden IPP und DMAP die Grundbausteine der Terpenbiosynthese.

Eine Alternative stellt der MEP-Weg (Abbildung 1.3.1, rechts) dar. Dabei wird IPP aus Pyruvat gebildet. Katalysiert von der Desoxyxylulosephosphat-Synthase (DXS) wird dieses mit Glycerinaldehyd-3-phosphat unter Abspaltung von Kohlenstoffdioxid verknüpft. Das entstandene 1-Desoxy-D-xylulose-5-phosphat (DXP) wird zum MEP isomerisiert, welches Namensgeber dieses Biogeneseweges ist. Die Cytidindiphosphat-Methylerythritol-Synthase (CMS) katalysiert die Verknüpfung des MEP mit Cytidindiphosphat unter Abspaltung des Pyrophosphats. Entstandenes 4-Diphosphocytidyl-2C-methyl-D-erythritol wird phosphoryliert und anschließend Cytidinmonophosphat enzymatisch gespalten und das Produkt cyclisiert. Resultierendes 2C-Methyl-D-erythritol-2,4-cyclodiphosphat wird enzymatisch unter Wasserabspaltung zu (*E*)-4-Hydroxy-3-methylbut-2-enyldiphosphat reduziert. Im finalen Schritt katalysiert die IPP/DMAPP-Synthase (IDS) die Bildung von IPP und DMAPP im Verhältnis 5:1 aus (*E*)-4-Hydroxy-3-methylbut-2-enyldiphosphat.

Katalysiert von einer Prenyltransferase, genannt Geranyldiphosphat-Synthase (GDS) kondensieren IPP und DMAPP zum Geranylpyrophosphat (GPP), welches der Vorläufer für die Monoterpene ist. Drei Untereinheiten bilden ein Farnesylpyrophosphat (FPP), welches den Grundbaustein der Sesquiterpene darstellt.

Sowohl Kopf-Kopf als auch Kopf-Schwanz, Schwanz-Schwanz sowie Kopf-Mitte Verknüpfungen des DMAPP mit dem IPP Untereinheiten sind bekannt (Buchanan *et al.* 2015). FPP entsteht durch Kopf-Schwanz-Verknüpfungen während Kopf-Kopf-Verknüpfungen von FPP und Geranylgeranylpyrophosphat zu C30 und C40 Terpenoiden führt (Scott *et al.* 2004).

Sesquiterpen-Synthasen (STS), auch Sesquiterpen-Cyclasen genannt, binden über das Mg^{2+} Cluster im aktiven Zentrum an die Pyrophosphatgruppe des FPP. Dabei orientiert sich die Prenylkette im hydrophoben katalytischen Zentrum. Es kommt zur Konformationsänderung, und der Pyrophosphatrest wird abgespalten (Davis und Croteau 2000). Daraus entsteht ein reaktives Carbokation. Sesquiterpen-Synthasen katalysieren eine Reaktionskaskade mit unterschiedlichen Cyclisierungsreaktionen und C-C-Knüpfungen, sodass sekundäre oder tertiäre Carbokation-Intermediate entstehen.

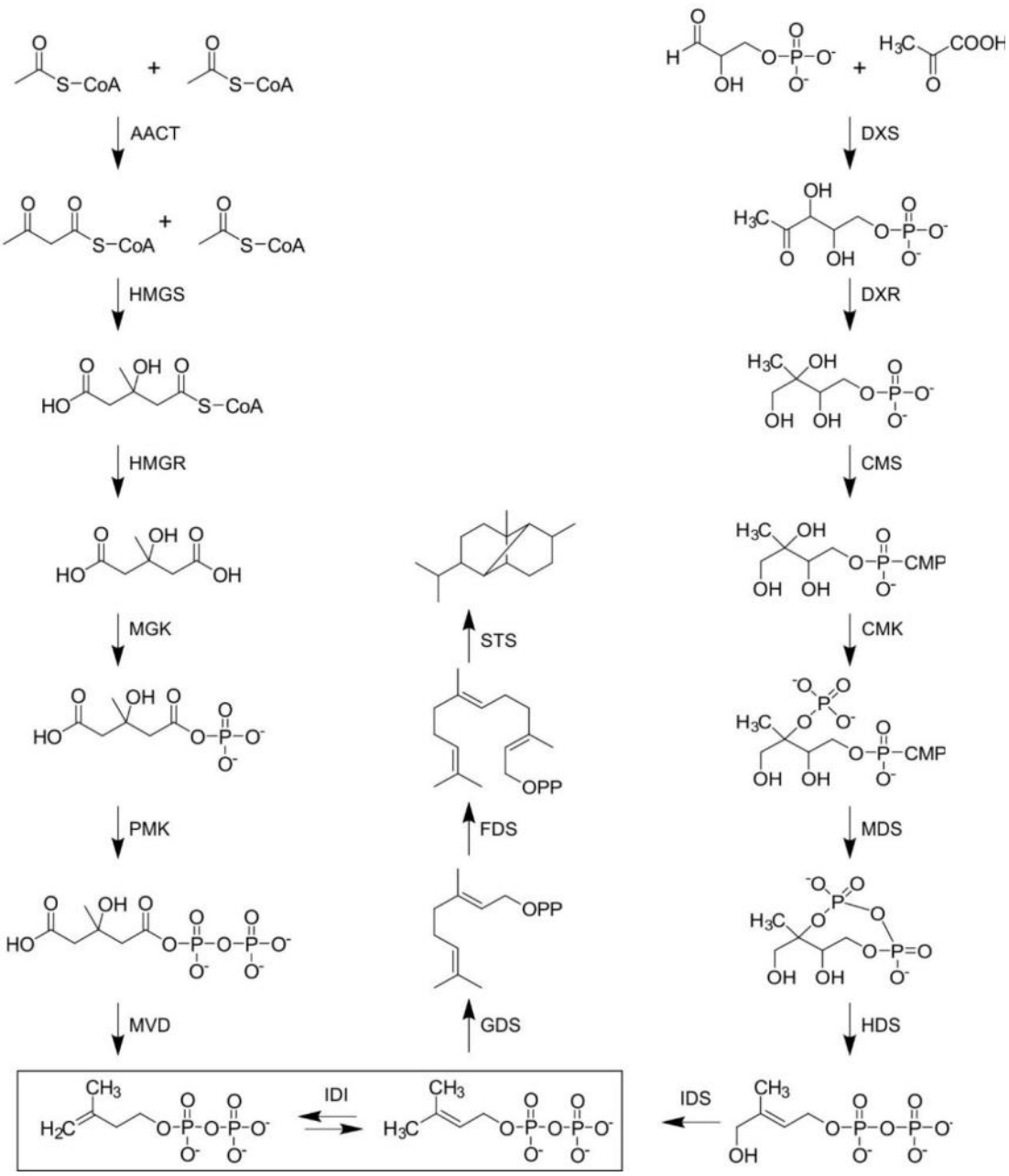


Abbildung 1.3.1. Darstellung der Sesquiterpen-Biosynthese über den MVA-Weg (links) und den MEP-Weg (rechts). Enzyme: Acetoacetyl-CoA-Thiolase (AACT), 3-Hydroxy-3-methylglutaryl-CoA-Synthase (HMGS), HMG-CoA-Reductase (HMGR), Mevalonat-Kinase (MGK), Phosphomevalonat-Kinase (PMK), PM-Decarboxylase (PMD), 1-Deoxy-D-xylose-5-phosphat-Synthase (DXS), DXP-Reductoisomerase (DXR), Cytidindiphosphat-Methylerythritol-Synthase (CMS), Cytidyl-Methyl-Kinase (CMK), Methyl-Erythritol-Cyclo-Diphosphat-Synthase (MDS), Hydroxy-Methyl-Butenyl-Diphosphat-Synthase (HDS), IPP/DMAPP-Synthase (IDS). Die Isopentyl-pyrophosphat-Isomerase (IPI) isomerisiert IPP zu DMAPP, beides Bausteile der Terpenbiosynthese. FPP wird über die Prenyltransferasen Geranyldiphosphat-Synthase (GDS) und Farnesyldiphosphat-Synthase (FDS) gebildet. Sesquiterpen-cyclasen (STS) katalysieren unter Abspaltung des Pyrophosphates die Cyclisierung und Umlagerung des FPP zu den unterschiedlichen Sesquiterpenen (frei nach George *et al.* 2015).

In Pilzen entstehen ausgehend vom *trans*-Farnesylcarbokation das *trans*-Humulylkation (1,11-Ringschluss) oder das *E*,*E*-Germacradienylkation (1,10-Ringschluss). Kommt es zur *cis-trans*-Isomerisierung, wird das Nerolidylcarbokation gebildet. Dieses cyclisiert zum Cycloheptenylkation (1,7-Ringschluss), zum (6*R*)-β-Bisabolylkation oder zum *Z*,*E*-Germacradienylkation (1,10-Ringschluss) (Schmidt-Dannert 2015). Das reaktive Carbokation wird im aktiven Zentrum der STS stabilisiert. Durch *Wagner-Meerwein*-Umlagerungen, Cyclisierungen, Hydrid-Shifts, und C-C-Knüpfungsreaktionen des sekundären Carbokations entsteht das finale Produkt (Davis und Croteau 2000; Schmidt-Dannert 2015; Miller und Allemann 2012). Dabei können tertiäre Carbokationen entstehen (Wedler *et al.* 2015). Bevor das Sesquiterpen aus dem katalytischen Zentrum entlassen wird, kommt es zur Deprotonierung oder zur Anlagerung von Wasser, gefolgt von einer Deprotonierung (Davis und Croteau 2000; Baer *et al.* 2014).

Produkte aus einem 1,10- und 1,6-Ringschluss dominieren das Produktspektrum von Basidiomyceten (Jansen und Groot 2004; Fraga 2013; Zheng *et al.* 2013). Dabei liegen der Mehrheit der Produkte Umlagerungen von Humulen zu Grunde (Abraham 2001). Zahlreiche Sesquiterpene können von einem Enzym produziert werden. Wie am Weißfäulepilz *Stereum hirsutum*, in welchem 18 Sesquiterpencyclasegene identifiziert wurden, gut erkennbar ist, produzieren Basidiomyceten meist nicht nur unterschiedliche Sesquiterpene, sondern auch mehr als eine STS (Quin *et al.* 2014). Somit ergibt sich ein breites Produktspektrum mit multiplen zellulären Regulationsmechanismen, welche das Volatilom grundlegend beeinflussen.

1.3.3 Ökologische Bedeutung basidiomycetischer Sesquiterpene

Lange wurden Sesquiterpene als metabolischer Müll oder Energiedepot angesehen (Gershenzon und Dudareva 2007). Erst in den frühen 2000ern änderte sich der Fokus

der Forschung hin zu der ökologischen Bedeutung dieser Verbindungen (Kramer und Abraham 2012). Heutzutage ist die Bedeutung der Sesquiterpene in der inter- sowie intraspezifischen Kommunikation bekannt. Rezeptorproteine wurden in Organismen aus unterschiedlichen Reichen beschrieben, welche auf eine Verwendung als Signalmolekül hindeutet (Unsicker *et al.* 2009). So wurde gezeigt, dass Insekten pilzliche Sesquiterpene wahrnehmen und darauf reagieren (Bruyne und Baker 2008). Weiterhin wurde auch die Kommunikation zwischen Pilzen unterschiedlicher Art gezeigt. Beispielsweise sekretieren *Resinicium bicolor* und *Hypholoma fasciculare* einen Cocktail aus α- und γ-Muurolen, α - und γ-Cadinen, α- und γ-Amorphen, sowie α- und γ-Bulgaren in räumlicher Nähe zueinander (Hynes *et al.* 2007).

Neben der Rolle als Signalmoleküle werden Sesquiterpene zur Abwehr sekretiert. Dabei soll die Sekretion einer Mischung von Sesquiterpenen nicht nur das Risiko der Resistenzausbildung verhindern, sondern auch einen breit aufgestellten Schutz gegen Fraßfeinde, Parasiten sowie Konkurrenten bieten (Anderson *et al.* 2010). So gelten unter anderem β-Farnesen, β-Humulen sowie α- und γ-Muurolen als Repellent gegen Herbivoren (Halls *et al.* 1994). Zusätzlich wurde β-Farnesen als Alarm-Pheromon in Blattläusen identifiziert (Kunert *et al.* 2005). Inoue *et al.* diskutierten ihre Wirkungsweise als Abwehrmechanismus, welchem die Bindung der lipophilen Sesquiterpene an der Zellmembran zur Destabilisierung des osmotischen Drucks zu Grunde liegt (Inoue *et al.* 2004). Weiterhin wird die Verwendung der unpolaren Sesquiterpene als Transporter für polare Zytotoxine diskutiert (Kramer und Abraham 2012).

Generell werden einem produzierten Sesquiterpencocktail multiple Effekte zugeschrieben. So produziert beispielsweise *Fusarium oxysporum*, welcher im

Konsortium mit Bakterien lebt, in Gegenwart von pathogenen Konkurrenten desselben Stamms Sesquiterpene. Dabei induziert *α*-Humulen eine Veränderung der pflanzenpathogenen Organismen hin zu einer nicht pathogenen Form. *β*-Caryophyllen wirkt als Wachstumsfaktor für die befallene Pflanze (Minerdi *et al.* 2011; Minerdi *et al.* 2009). Die genannten Beispiele zeigen die Komplexität der ökologischen Funktion dieser Substanzklasse. Bekannt ist, dass die volatilen Verbindungen als Signalmoleküle wirken. Sowohl anziehende als auch abwehrende Effekte wurden in den letzten Jahrzehnten beschrieben. Derzeit stellt das Bekannte einen Flickenteppich aus Einzelfällen dar. Kramer und Abraham (2012) vermuten eine sensible Kommunikation zwischen Pilzen, Pflanzen und Insekten beeinflusst durch die Umgebung dieser Organismen. Systematische Ansätze zur Beschreibung fehlen jedoch.

2 Zielsetzung der Arbeit

Verbraucher fordern verstärkt eine nachhaltige Produktion natürlicher Aromastoffe. Gleichwohl bietet die Biotechnologie ressourcenschonende, skalierbare und enantioselektive Prozesse mit milden Reaktionsbedingungen. Speziell Basidiomyceten haben ein großes Potential aufgrund der Ähnlichkeit zum pflanzlichen Volatilom. Sie gelten als wenig erforschte Organismen und bieten daher ein hohes Potential für die Identifizierung von neuartigen Aroma- und Naturstoffen sowie von Enzymen mit außergewöhnlichen Eigenschaften. Insbesondere die *de-novo*-Synthese acetogener Aromastoffe befindet sich im Fokus. Gesteigertes Verständnis grundlegender Biogenesewege, sowie von Effektoren erlaubt ein gezieltes Design von Expressionskassetten und Prozessen zur heterologen Produktion von natürlichen Aromen im Ganzzellexpressionssystem. Dabei stellen Polyketide und Terpenoide die beiden größten Gruppen basidiomycetischer Acetoide dar.

Ziel dieser Arbeit war die Identifizierung eines *de-novo* produzierten Ananasaromas aus dem Kulturüberstand von *Fomitopsis betulina*. Dies beinhaltet die eindeutige Identifizierung des Naturstoffs über eine Chemosynthese sowie die sensorische Charakterisierung dieses neuen Aromastoffs. In weiterführenden Arbeiten sollte die Produktion von *F. betulina* in Submerskultur charakterisiert sowie die Biogenese geklärt werden.

Weiterhin sollte die Produktion von Acetoiden besser verstanden werden. Für den Modellorganismus *Tyromyces floriformis* sollten Effektoren der Biosynthese identifiziert werden. Sowohl die Änderungen des Produktspektrums als auch Konzentrationsänderungen waren im Fokus. Beobachtete Effekte sollten verstanden werden, um Rückschlüsse auf intrazelluläre Prozesse ziehen zu können. Ferner sollte die Übertragbarkeit identifizierter Effekte auf andere Basidiomyceten geprüft werden.

3 (5*E*/*Z*,7*E*,9)-Decatrien-2-ones, Pineapple-like Flavors from *Fomitopsis betulina* - Structure Elucidation and Sensorial Properties

3.1 Vorwort zur Publikation

Die Identifizierung neuer Aromastoffe ist eine stetige Herausforderung der modernen Biotechnologie. In Vorarbeiten wurde von Silke Schimanski ein starker Geruch nach Ananas bei der Kultivierung von *Fomitopsis betulina* auf Weißkohl wahrgenommen. Gaschromatographische Analysen gekoppelt mit Olfaktometrie identifizierten zwei Substanzen mit identischem Massenspektrum, was auf die Präsenz zweier Stereoisomere hindeutete (Schimanski *et al.* 2013).

Ananas erfreut sich unter den exotischen Früchten besonderer Beliebtheit. Da diese Frucht selbst keinen Schlüsselaromastoff besitzt, warf die Identifizierung eines durch eine einzige Verbindung ausgelösten Geruchseindrucks nach Ananas die Frage nach den zu Grunde liegenden Eigenschaften auf.

Diese Arbeit zeigt die Identifizierung eines neuen Naturstoffs aus dem Kulturüberstand des Birkenporlings. Weiterhin gibt die Arbeit Aufschluss über die Charakteristika, welche einen ananasartigen Geruchseindruck hervorrufen.

Tim Pendzialeck führte für eine eindeutige Produktidentifizierung im Rahmen seiner Masterarbeit die Retrosynthese des (5*E*/*Z*,7*E*,9)-Decatrien-2-on und seiner Analoga durch und charakterisierte das Aroma sowie die Abhängigkeit zwischen Geruchseindruck und Struktur. Jörg Fohrer führte die Strukturaufklärung mittels NMR durch. Ralf G. Berger stellte die Projektidee sowie nötige Mittel zur Verfügung. Ulrich Krings übernahm die Interpretation der Massenspektren sowie die wissenschaftliche Betreuung.

Bei der nachfolgenden Arbeit handelt es sich um ein akzeptiertes Manuskript publiziert bei ACS Publications im *Journal of Agricultural and Food Chemistry* am 25.11.2019, verfügbar online unter https://doi.org/10.1021/acs.jafc.9b06105.

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(5*E*/*Z*,7*E*,9)-Decatrien-2-ones, Pineapple-like Flavors from *Fomitopsis betulina*—Structure Elucidation and Sensorial Properties

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3.2 Abstract

During the cultivation of the edible mushroom *Fomitopsis betulina* on agro-industrial side streams, a pleasant flavor strongly reminiscent of pineapple was perceived. Aroma extract dilution analyses identified two flavor components with a distinct pineapple odor. On the basis of mass spectrometric data, a Wittig reaction of (*E*)-penta-2,4-dien-1-yltriphosphonium bromide with ethyl levulinate was conducted. The resulting (5*E*/*Z*,7*E*,9)-decatrien-2-ones were identical to the compounds isolated from the fungal culture. Some structurally related methyl ketones were synthesized, confirmed by nuclear magnetic resonance and mass spectrometry, and their odor was characterized. The lowest odor threshold and most characteristic pineapple-like odor was found for (5*Z*,7*E*,9)-decatrien-2-one. Global minimum energy calculation of the methyl ketones and the comparison to (1,3*E*,5*Z*)-undecatriene, a character impact compound of fresh pineapple, showed that a chain length of at least 10 carbon atoms and a terminal double bond embedded in a “L”-shaped conformation were common to compounds imparting an intense pineapple-like odor. Both (5*E*/*Z*,7*E*,9)-decatrien-2-ones have not been described as natural flavor compounds.

Keywords: pineapple flavor, (5*E*/*Z*,7*E*,9)-decatrien-2-ones, structure−activity relationship

3.3 Introduction

Pineapple is one of the most popular tropical fruits because of its unique flavor. More than 380 volatiles, including esters, heterocycles, and polyunsaturated hydrocarbons, were reported (Montero-Calderón und Rojas-Graü, M. A. and Martín-Belloso O. 2010; Belitz *et al.* 2008; Steingass *et al.* 2015). The key aroma compounds are 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, ethyl-2-methylbutanoate, ethyl-2-methylpropanoate, methyl-2-methylbutanoate, and (1*E*/*Z*,3,5)-undecatriene (Belitz *et al.* 2008). Takeoka et al. stated that, besides the key aroma compounds, ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl 2-methylpropanoate, methyl hexanoate, and methyl butanoate are required to create the exotic sweet flavor of fresh pineapple, whereas Pino stated that solely the addition of ethyl 3-(methylthio)propanoate and (1,3*E*,5*Z*,8*Z*)-undecatetraene was required (Takeoka *et al.;* Pino 2013; Berger *et al.* 1985). However, a characteristic genuine impact compound, such as vanillin for vanilla, has not yet been identified.

During the cultivation of numerous basidiomycetous fungi on various agro-industrial side streams, the formation of a pleasant flavor, strongly reminiscent of pineapple and honey, was perceived in cell cultures of the edible mushroom *Fomitopsis betulina* (formerly *Piptoporus* or *Polyporus betulinus*) when grown on cabbage cuttings. Aroma extract dilution analyses resulted in the identification of six important flavor components, among them one with a distinct pineapple odor (Schimanski *et al.* 2013). Hence, the fungi generated an aroma compound that evoked a strong pineapple odor impression on its own.

The aims of this study were to elucidate the unknown chemical structure of the flavor compounds based on total synthesis, determine their sensory properties, and model the structure-activity relationship based on analogues synthesized using the same

route. This work not only identified a new impact aroma compound but also contributes to the understanding of pineapple flavor perception.

3.4 Materials and Methods

3.4.1 Chemicals and Substances

Chemicals used were purchased in pro analysi (p.a.) quality from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). Dec-9-en-2-one was from Sigma-Aldrich (St. Louis, MO, U.S.A.) in a quality > 95 % and used without further purification. All solvents were purified in house by rectification.

3.4.2 Synthesis of (5*E*/*Z*,7*E*,9)-Decatrien-2-ones and Structural Related Compounds

(5*E*)-Bromopenta-1,3-diene (1). To a hydrogen bromide (HBr) solution (3.4 mL, 48 % (v/v), 30.3 mmol) at 5 °C penta-1,4-dienol (2.50 g, 98 %, 29.1 mmol) was added dropwise within 5 min, and the reaction mixture was stirred for another 3 h at room temperature (RT). During this time, diethyl ether (8 mL) and another portion of BHr were added. Afterward, the organic layer was separated, washed with cold water (3 × 10 mL), and dried over magnesium sulphate ($MgSO_4$). After filtration, the solvent was removed under reduced pressure using a rotary evaporator. The product (3.82 g, 26.0 mmol, 89 % yield) was obtained as a yellow oil.

Retention index (RI): 1250 (DB-WAX UI) and 885 (VF-5 ms).

Electron impact mass spectrometry (EI–MS) (C_5H_7Br, M = 147 g mol^{-1}): *m/z* (%) = 148 ($M^{+\bullet}$, 11), 146 ($M^{+\bullet}$, 11), 94 (1), 92 (1), 81 (2), 79 (2), 67 (100), 65 (15), 51 (4), 41 (39) and 39 (25).

(*E*)-Penta-2,4-dien-1-yltriphenylphosphonium Bromide (2). (5*E*)-Bromopenta-1,3-diene (5.18 g, 35.2 mmol) was added dropwise to a mixture of triphenylphosphine [9.85 g 98.5 % (*v/v*), 37.0 mmol] dissolved in toluene (23 mL) under a nitrogen

atmosphere. After stirring the mixture at RT for 22 h crystalline (*E*)-penta-2,4-dien-1-yltriphenylphosphonium bromide was separated *via* filtration (10.75 g, 26.3 mmol, 75 % yield) as a colorless solid.

Positive electrospray ionization tandem mass spectrometry [ESI(+)–MS/MS] ($C_{23}H_{22}P$, M^+ = 329 g/mol): *m/z* (%) = 329 (M^+, 10), 262 (100), 185 (13), 183 (50), 108 (87), 107 (6) and 67 (13).

Ethyl-3-(2-methyl-1,3-dioxolan-2-yl)propanoate (3). A solution of ethyl levulinate (10.0 g, 69.4 mmol), ethylene glycol (7.0 mL, 125.5 mmol), and *p*-toluenesulfonic acid monohydrate (0.13 g, 0.7 mmol) in cyclohexene (40 mL) was heated in a Dean-Stark apparatus for 4 h under reflux. After cooling to RT, saturated sodium hydrogen carbonate solution ($NaHCO_3$) (20 mL) was added. The organic phase was washed with water (3 × 20 mL) and dried over $MgSO_4$. The solvent was removed with a rotary evaporator and the product (5.41 g, 91 % purity, 28.7 mmol, 41 % yield) was obtained as a colorless oil (Miyazawa *et al.* 2009a).

EI–MS ($C_9H_{16}O_4$, M = 188 g/mol): *m/z* (%) = 173 (18), 143 (30), 129 (4), 99 (48), 87 (100), 45(3) and 43 (35).

3-(2-Methyl-1,3-dioxolan-2-yl)propanal (4). Under a N_2-atmosphere, acetal (**3**) (2.50 g, 13.3 mmol) was dissolved in dry dichloromethane (DCM, 74 mL) at –78 °C. To this solution, 13.9 mL of 1 M diisobutyl aluminumhydride (DIBAL, 13.9 mmol) in *n*-hexane was added dropwise. After 45 min under continuous stirring at –78 °C, 2.5 mL of methanol (MeOH) and 10.0 mL of saturated sodium chloride solution (NaCl) were added and stirred for another 1 h at the same temperature. Then, the reaction mixture was stirred overnight at RT. After the addition of 12.5 g of Na_2SO_4 and stirring for 1 h, the organic phase was filtered, the filter was washed with DCM (3 × 10 mL) and finally

the solvent was removed with the rotary evaporator. The residue was purified by flash chromatography [silica gel, elution with pentene/diethyl ether (PE; 1:1.12, *n/n*) and diethyl ether (DE), PE 3:1 → DE]. The purified product (1.13 g, 7.8 mmol, 59 % yield) was obtained as a colorless oil (Ogura und Usuki 2013).

RI: 1623 (DB-WAX UI) and 1079 (VF-5 ms).

EI–MS ($C_7H_{12}O_3$, M = 144 g/mol): *m/z* (%) = 129 (27), 87 (100), 85 (26), 71 (6), 57 (7), 55 (11) and 43 (64).

2-Methyl-2-((5*E*)-octa-3,5,7-trien-1yl)-1,3-dioxolane (5a and **5b).** Under a N_2-atmosphere, aldehyde (**4**) (0.50 g, 3.5 mmol) and phosphonium salt (**2**) (1.56 g, 3.8 mmol) were dissolved in dry dimethylformamide (DMF, 8 mL). After dropwise addition of sodium methanolate (0.7 mL, 30 % in methanol, 3.6 mmol) at 5 °C, the reaction mixture was stirred for 2 h at a constant temperature. Saturated ammonium chloride solution (NH_4Cl, 30 mL) and *n*-hexane (30 mL) were added prior to filtration. The organic phase of the filtrate was separated, and the aqueous phase was extracted with *n*-hexane (3 × 30 mL). The combined organic phase was washed with water and saturated NaCl-solution (3 × 30 mL each), dried over $MgSO_4$, and then mixed with butylated hydroxyl toluene (BHT, 10 mg) for stabilization. The solvent was removed with a rotary evaporator under reduced pressure, and the residue was purified by flash chromatography (silica gel, elution PE, PE 20:1 → PE 8:1). The purified product (0.51 g, 2.6 mmol, 76 % yield) was obtained as a light yellow oil (Miyazawa *et al.* 2009a).

3(*Z*) Isomer (**5b**). RI: 1948 (DB-WAX UI) and 1462 (VF-5 ms).

EI–MS: ($C_{12}H_{18}O_2$, M = 194 g/mol) *m/z* (%) = 194 ($M^{+\bullet}$, 19), 179 (7), 93 (19), 91 (35), 87 (100), 79 (16), 77 (27) and 43 (75).

3(*E*) Isomer (**5a**). RI: 1991 (DB-WAX UI) and 1487 (VF-5 ms).

EI–MS: ($C_{12}H_{18}O_2$, M = 194 g/mol): *m/z* (%) = 194 ($M^{+\bullet}$, 22), 179 (7), 93 (23), 91 (39), 87 (100), 79 (16), 77 (30) and 43 (78).

(5 *E*/*Z*,7*E*,9)-Decatrien-2-one (6a and **6b).** To a mixture of 3 mL of perchloric acid ($HClO_4$, 35 %) and 1 mL of DE at 5 °C, 2 mL of a solution of acetal (**5a** and **5b**) in DE (0.20 g, 1 mmol) was added dropwise within 10 min. After 20 min at a constant temperature (5 °C), the reaction mixture was neutralized with saturated $NaHCO_3$–solution. The organic phase was separated, and the aqueous phase was extracted with DE (3 × 20 mL). The combined organic phase was washed with water and saturated NaCl–solution (3 × 30 mL), dried over $MgSO_4$, and stabilized with BHT (10 mg). The residue was purified by flash chromatography (silica gel, elution with PE 10:1). The purified product (57 mg, 0.38 mmol, 37 % yield) was obtained as a colorless oil (Miyazawa *et al.* 2009a).

5(*Z*) Isomer (**6b**). RI: 1826 (DB-WAX UI) and 1268 (VF-5 ms).

EI–MS ($C_{10}H_{14}O$, M = 150 g/mol): *m/z* (%) = 150 ($M^{+\bullet}$, 35), 135 (1), 107 (16), 93 (14), 91 (81), 79 (57), 71 (3), 67 (11), 65 (18) and 43 (100).

5(*E*) Isomer (**6a**). RI: 1846 (DB-WAX UI) and 1278 (VF-5 ms).

EI–MS ($C_{10}H_{14}O$, M = 150 g/mol): *m/z* (%) = 150 ($M^{+\bullet}$, 37), 135 (1), 107 (16), 93 (15), 91 (81), 79 (55), 71 (3), 67 (11), 65 (18) and 43 (100).

^{1}H-NMR (6a and **6b):** (400 MHz, $CDCl_3$): δ 2.14, 2.14 ($s_{a,b}$, 6H, $1_{a,b}$), 2.35-2.49 (m, 4H, $4_{a,b}$), 2.51-2.55 (m, 4H, $3_{a,b}$), 5.05, 5.10 ($d_{a,b}$, J_1 = 10.09 Hz, J_2 = 10.25 Hz, 2H, $10_{a,b}$), 5.18, 5.23 ($d_{a,b}$, J_1 = 16.70 Hz, J_2 = 16.82 Hz, 2H, $10_{a,b}$), 5.42 (dt, J_1 = 7.47 Hz,

J_2 = 10.68 Hz, 1H, 5_b), 5.69 (dt, J_1 = 7.01 Hz, J_2 = 14.76 Hz, 1H, 5_a), 6.01- 6.03 (m, 8H, $6_{a,b}$, $7_{a,b}$, $8_{a,b}$, $9_{a,b}$).

^{13}C-NMR (6a and **6b):** (100 MHz, $CDCl_3$): δ 22.3, 27.0, 30.1, 43.2, 43.5, 116.9, 117.6, 128.2, 129.5, 130.9, 131.2, 133.2, 133.6, 133.9, 137.1, 137.2, 208.06, 208.14.

5,9-Decadien-2-one (7a and **7b).** This compound was synthesized along the same route as the decatrienones (**6a** and **6b**) with pent-4-en-1-yltriphosphonium bromide used in the Wittig reaction. The product (100 mg, 0.6 mmol, 53 % yield) was obtained as a colorless oil.(Miyazawa *et al.* 2009a)

5(*Z*) Isomer (**7b**). RI: 1582 (DB-WAX UI) and 1171 (VF-5 ms).

EI–MS ($C_{10}H_{16}O$, M = 152 g/mol): *m/z* (%) = 152 ($M^{+\bullet}$, 1), 111 (5), 109 (2), 97 (1), 94 (14), 81 (2), 79 (13), 71 (4), 43 (100) and 41 (12).

5(*E*) Isomer (**7a)**. RI: 1173 (VF-5 ms).

EI–MS ($C_{10}H_{16}O$, M = 152 g/mol): *m/z* (%) = 152 ($M^{+\bullet}$, 1), 111 (5), 109 (2), 97 (1), 94 (12), 81 (2), 79 (10), 71 (3), 43 (100) and 41 (10).

5-Decen-2-one (8a and **8b).** This compound was synthesized along the same route as the decatrienones (**6**), with pentyltriphosphonium bromide used in the Wittig reaction. The product (70 mg, 0.45 mmol, 67 % yield) was obtained as a colorless oil (Miyazawa *et al.* 2009a).

5(*Z*) Isomer (**8b)**. RI: 1530 (DB-WAX UI) and 1179 (VF-5 ms).

EI–MS ($C_{10}H_{16}O$, M = 152 g/mol): *m/z* (%) = 152 ($M^{+\bullet}$, 1), 111 (5), 109 (2), 97 (1), 94 (14), 81 (2), 79 (13), 71 (4), 43 (100) and 41 (12).

5(*E*) Isomer (**8a**). RI: 1183 (VF-5ms).

EI–MS ($C_{10}H_{16}O$, M = 152 g/mol): *m/z* (%) = 152 ($M^{+\bullet}$, 1), 111 (5), 109 (2), 97 (1), 94 (12), 81 (2), 79 (10), 71 (3), 43 (100) and 41 (10).

(5,7*E*)-Nonadien-2-one (9a and **9b).** This compound was synthesized along the same synthetic route as the decatrienones (**6**) with (*E*)-buten-2-en-1-yltriphosphonium bromide used in the Wittig reaction. A product mixture of compounds **9a** and **9b** (80 mg, 81 % purity, 0.47 mmol, 59 % yield) and its constitutional isomer, 6-methylocta-5,7(*E*)-dien-2-one (**10a** and **10b)** (80 mg, 16 % purity, 0.092 mmol, 59 % yield) was obtained as a light yellow oil (Miyazawa *et al.* 2009a).

5(*Z*) Isomer (**9b**). RI: 1596 (DB-WAX UI) and 1139 (VF-5 ms).

EI–MS ($C_9H_{14}O$, M = 138 g/mol): *m/z* (%) = 138 ($M^{+\bullet}$, 29), 123 (5), 95 (41), 81 (31), 79 (38), 67 (29), 55 (21) and 43 (100), 41 (22).

5(*E*) Isomer (**9a**). RI: 1605 (DB-WAX UI) and 1145 (VF-5 ms).

EI–MS ($C_9H_{14}O$, M = 138 g/mol): *m/z* (%) = 138 ($M^{+\bullet}$, 31), 123 (5), 95 (40), 81 (32), 79 (38), 67 (28), 55 (20), 43 (100) and 41 (21).

5(*Z*) Isomer (**9c**). RI: 1613 (DB-WAX UI) and 1150 (VF-5 ms).

EI–MS ($C_9H_{14}O$, M = 138 g/mol): *m/z* (%) = 138 ($M^{+\bullet}$, 27), 123 (5), 95 (40), 81 (29), 79 (38), 67 (27), 55 (20), 43 (100) and 41 (21).

5(*E*) Isomer (**9d**). RI: 1617 (DB-WAX UI) and 1154 (VF-5 ms).

EI–MS ($C_9H_{14}O$, M = 138 g/mol): *m/z* (%) = 138 ($M^{+\bullet}$, 23), 123 (5), 95 (40), 81 (28), 79 (37), 67 (27), 55 (20), 43 (100) and 41 (21).

5,7-Octadien-2-one (10a and **10b).** This compound was synthesized along the same route as the decatrienones (**6**), with allyltriphosphonium bromide used in the Wittig

reaction. The product (80 mg, 0.47 mmol, 59 % yield) was obtained as a colorless oil (Miyazawa *et al.* 2009a).

5(*Z*) Isomer (**10b**). RI: 1463 (DB-WAX UI).

5(*E*) Isomer (**10a**). RI: 1465 (DB-WAX UI).

EI–MS (*E*) und (*Z*) isomers ($C_8H_{12}O$, M = 124 g/mol): *m/z* (%) = 124 ($M^{+\bullet}$, 12), 109 (5), 81 (33), 79 (22), 71 (1), 67 (20), 53(12) and 43 (100).

3.4.3 Purification of (5*E*,7*E*,9)-Decatrien-2-one (6a)

Product purification was performed via preparative gas chromatography (GC). A Hewlett-Packard 5890 (Hewlett-Packard, Palo Alto, CA, U.S.A.) gas chromatograph was equipped with a HP 7673 auto sampler (Hewlett-Packard, Palo Alto, CA, U.S.A.), a cold injection system (CIS 3, Gerstel, Mülheim, Germany) and a multi column switching system II (Gerstel, Mülheim, Germany). Separation was performed on a polar Optima-Wax precolumn (5 m, 0.53 mm, 2 µm, Macharey-Nagel Düren, Germany) and an Optima Wax preparative column (25 m, 0.53 mm, 2 µm, Macherey-Nagel Düren, Germany) with a hydrogen flow of 8.9 mL/min. For separation, the following temperature program was used: 40 °C held for 3 min, 1 °C min^{-1} until 168 °C, 20 °C min^{-1} until 240 °C, and held for 15 min. The product was collected with the multi-column switching system II. The purified product was used for nuclear magnetic resonance (NMR) characterization.

3.4.4 Gas Chromatography–Mass Spectrometry (GC–MS)

All products synthesized with the exception of the respective Wittig salts were diluted to an appropriate concentration with diethyl ether and analyzed by means of high-resolution capillary gas chromatography coupled to a mass selective detector (GC–MS). GC analyses were performed on two stationary phases of different polarity.

Retention indices were calculated according to Van den Dool and Kratz using homologue alkanes from C8 to C30. Samples (0.5 µL) were injected on-column (van den Dool und Dec. Kratz 1963). The GC–MS device consisted of a 7890B GC–system (Agilent Technologies, Santa Clara, CA, U.S.A.) combined with an Agilent 5977A mass selective detector (interface, 230 °C; ion source, 200 °C; quadrupole, 100 °C; electron impact ionization, 70 eV; scan range, *m/z* 33–300 amu; Agilent Technologies, Santa Clara, CA, U.S.A.). Stationary phases DB-WAX UI (30 m, 0.25 mm, 0.25 µm, Agilent J&W GC Columns, Santa Clara, CA, U.S.A.) and VF-5 ms (30 m, 0.25 mm, 0.25 µm, Agilent J&W GC columns, Santa Clara, CA, U.S.A.) were installed. All analyses were performed with a volumetric flow rate of 1 mL/min helium using the following temperature programs: 40 °C held for 3 min, 8 °C/min until 230 °C, and 230 °C held for 10 min (DB-WAXms) and 40 °C held for 3 min, 8 °C/min until 230 °C, 25 °C/min until 325 °C, and 325 °C held for 10 min (VF 5 ms).

High–resolution GC was performed with an Agilent 6890 gas chromatograph equipped with a Quasar AMD (electron impact ionization, 70 eV; scan range, *m/z* 33–500 amu; Agilent Technologies, Santa Clara, CA, U.S.A.). Separation was performed on a DB-WAX column (30 m, 0.32 mm, 0.25 µm, Agilent J&W GC columns, Santa Clara, CA, U.S.A.) using the conditions described above.

3.4.5 Gas Chromatography–Olfactometry (GC–O)

Sensory evaluation and determination of the perception threshold mass of the decatrien-2-ones were carried out using GC-O combined with aroma extract dilution analysis (AEDA). The same chromatographic conditions were used as described but with hydrogen as the carrier gas. The effluent was split at the end of the column (1:1). One part was directed toward a flame ionization detector (FID), and the second part was directed toward the olfactory detection port (ODP), where the effluent was sniffed

by panelists (five trained subjects). The stock solution contained all synthesized compounds and commercial 9-decen-2-one (0.1 g/L in DE). Sensory evaluation started with a 1:10 dilution of the stock solution and was continued with further 1:10 dilution steps until no odor of any of the flavor compounds was perceived. The individual thresholds were expressed as the ultimate mass (m_T) injected effectively on-column that was still perceived by the majority of the panelists. The median of the individual thresholds was calculated.

3.4.6 High Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS²)

The synthesis of Wittig salts was confirmed using high–performance liquid chromatography coupled to a triple quadrupole mass analyzer (Varian 212 LC pump, Pro Star 325 UV/vis detector, and 320 TQ-MS mass spectrometer). MS was conducted in the positive electron spray ionization [ESI(+)] mode with the selective mass of the respective Wittig cation for the first quadrupole and a scan range of *m/z* 50 to the respective mass of the cation in the third quadrupole. MS parameters were as follows: capillary voltage, +40 V; needle voltage, 5000 V; nebulizer gas (N_2), 379 kPa; and drying gas, 207 kPa, at 350 °C. The collision cell was operating with 267 mPa argon as the collision gas and 40 V collision energy. For HPLC, water and acetonitrile (MS-grade), both containing 0.1 % formic acid, were used as the mobile phase. The following linear gradient was used: 10 % acetonitrile for 3 min, up to 90 % acetonitrile within 20 min, hold for 5 min, and back to the starting conditions. The separation was performed on a Nucleodur C18 HD Pyramid–column (250×4 mm, 5 µm, Macherey-Nagel) at a flow rate of 0.3 mL/min. Samples were dissolved in acetonitrile/water (1:1), diluted to appropriate concentrations, and 20 µL was injected manually *via* a six–port valve.

3.4.7 Structure-Activity Relationship

The compounds used in the olfactory analysis were compared to the structure of the genuine pineapple impact aroma volatiles (1,3*E*,5*Z*)-undecatriene and (1,3*E*,5*Z*,8*Z*)-undecatetraene. To identify the most abundant molecular conformation of each compound, structures were energy-minimized using the DREIDING force field. These data were used for quenched dynamics simulation (1 ns, 300 K) to obtain the most likely geometric spatial conformation of the odor compounds (Mayo *et al.* 1990).

3.4.8 NMR Spectroscopy

Structure elucidation was performed by ^{1}H and ^{13}C one-dimensional (1D) spectra with broadband decoupling as well as two-dimensional (2D) spectra, such as double quantum filtered correlation spectroscopy (DQF-COSY) for proton correlations and ^{13}C-edited heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectra for proton carbon correlations. All measurements were performed on a Bruker Ultrashield 500 MHz spectrometer with a DRX console and cryo TCI ^{1}H, $^{13}C/^{15}N$ probe with *z* gradients. For measurements, the products were dissolved in 0.7 mL of deuterated trichloromethane and measured at 298.0 K. The residual solvent signal of trichloromethane was used as an internal standard. All data were evaluated by MestReNova and TopSpin software.

3.5 Results and Discussion

3.5.1 Retrosynthesis of (5*E*/*Z*,7*E*,9)-Decatrien-2ones

The retrosynthesis of target (5*E*/*Z*,7*E*,9)-decatrien-2-ones is shown in Figure 3.5.1. The decatrien-2-one skeleton was built along a Wittig reaction of two C5 units, an aldehyde with a methyl ketone group and a triphenylphosphonium salt bearing the conjugated pentadiene unit. To avoid side reactions, unprotected 4-ketopentanal was not reacted directly with the respective Wittig salt. Thus, the keto group was converted

to its acetal (**3**). Subsequently, a reductive acetal deprotection (DIBAL) was required after the Wittig reaction to yield targeted decatrien-2-ones (**6a** and **6b**).

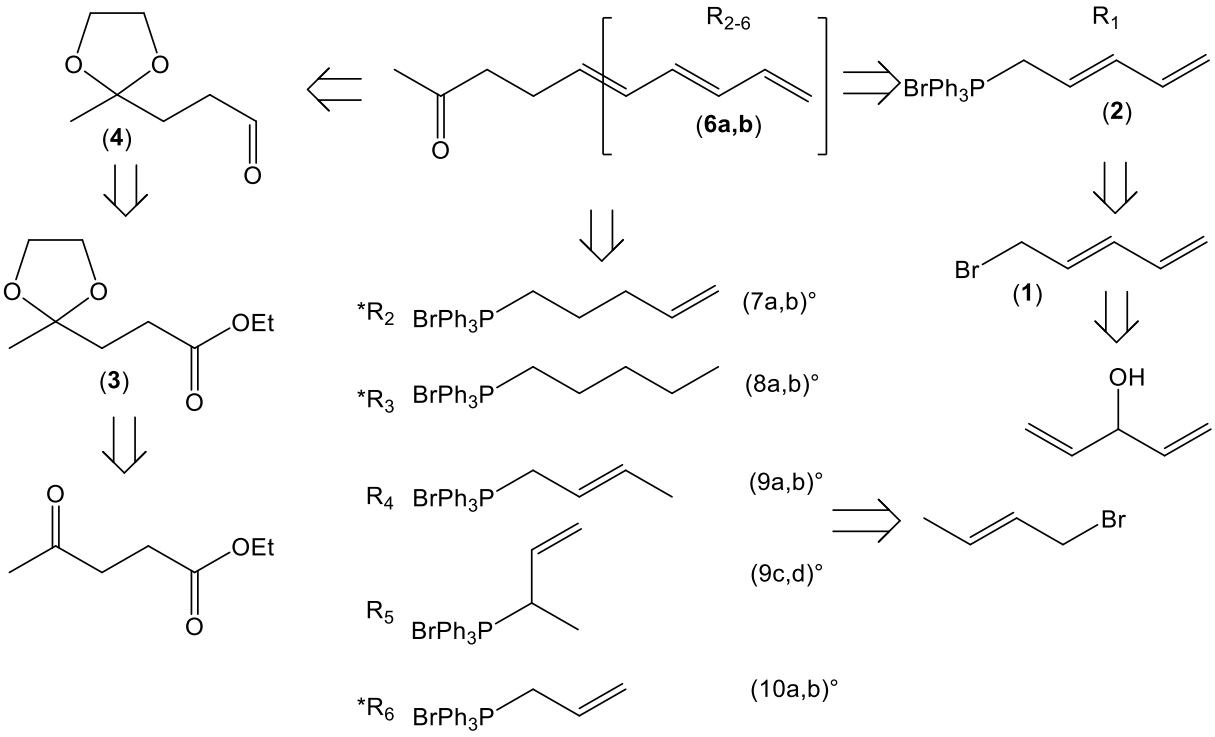


Figure 3.5.1. Retrosynthesis of (5*E*/*Z*,7*E*,9)-decatrien-2-one and structurally related methyl ketones (numbering corresponds to table 3.5.1; * = commercial product; ° number of the respective final product).

The triene moiety of decatrien-2-one was introduced *via* phosphonium salt of (1,3*E*)-pentadiene, which was generated by the nucleophilic substitution (S_N1') of 1,4-pentadienol with HBr. This reaction proceeds stereoselectively under the formation of 1,3(*E*)-conjugated double bonds. The second C5–unit for the Wittig reaction, aldehyde (**4**), providing the methyl ketone unit, was synthesized starting with ethyl levulinate. The keto group was protected by acetal formation with ethylene glycol, and the ester was subsequently reduced with DIBAL to give aldehyde (**4**).

Both C5–units were coupled in a Wittig reaction, resulting in the targeted C10 skeleton and the third double bond in conjugation to the already present conjugated diene system. In general, the (*E/Z*) selectivity of the Wittig reaction depends upon the stabilization of the mesomeric ylide and ylene intermediates. Stabilization of the ylide favors the formation of the (*E*) isomer, whereas non-stabilization gives the (*Z*) isomer. In the case of preferred ylene formation in the transition state, bulky substituents favor the formation of the (*Z*) isomer, whereas repellant electronegative groups favor the formation of the (*E*) isomer. Here, no preference for one of the 2-(5(*E*)-hepta-3,5-dien-1-yl)-2-methyl-1,3-dioxolane isomers [**5a** and **5b**, (*E/Z*) = 44:56; assignment by ^{1}H–NMR] was found. Hence, after removal of the protection group, targeted (5*E/Z,*7*E*,9)-decatrien-2-ones (**6a** and **6b**) were obtained with an almost equimolar isomeric distribution.

Separation of the two isomers succeeded *via* GC–MS analysis (CW20M, 1842 and 1863). However, an assignment to the respective 5(*E*/*Z*) on the basis of the electron impact (EI) mass spectra was not possible. Re-evaluation of extracts from the fungal culture supernatant at the day of maximum product concentration [1.1 mg/L 5(*E*) isomer and 0.07 mg/L 5(*Z*) isomer, at day 5] revealed the presence of both compounds. The mass spectra of the synthesized isomers were nearly identical to each other and to the mass spectra of the pineapple flavor compounds found in culture medium of the basidiomycete *F. betulina*.

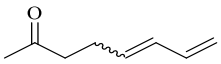
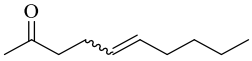
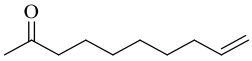
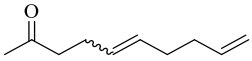
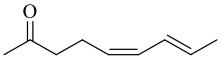
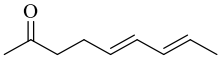
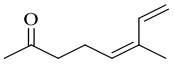
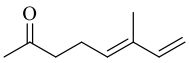
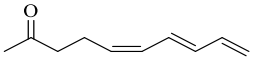
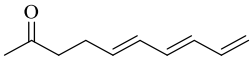
3.5.2 Structure Elucidation of (5*E*/*Z*,7*E*,9)-Decatrien-2-ones

Both isomers were clearly separated on GC–columns of different polarity, but the EI-mass spectra showed no significant differences. Ionization at carbonyl oxygen resulted in neutral losses of *m/z* 15 (CH_3) and *m/z* 43 ($CH_3C{=}O$), indicative of the terminal methyl ketone group. As soon as the conjugated triene moiety of the molecule

becomes ionized, fragmentation occurs preferentially after rearrangements. The intensity of the signals at *m/z* 91/92 and 77/79 indicated the formation of stable cyclic ions as they are known for cyclohexene derivatives, such as limonene. A similar behavior was observed while analyzing polyenes, *inter alia* (1,3,5)-undecatriene. However, due to the ionization of the conjugated triene system, information about the stereochemistry of the double bonds (Δ5 and Δ7) was lost.

Accurate mass determination resulted 150.096 amu, corresponding to an empirical formula of $C_{10}H_{14}O$ (150.104 calculated). The pineapple-like smelling diastereomers were identified as (5*E*/*Z*,7*E*/*Z,9*)-decatrien-2-ones, but the geometry of the Δ5 and Δ7 double bonds remained unknown. Data on decatrien-2-ones are rare in the literature. In 2010, Nakanishi and Watanabe published a patent and Tomita published a research article dealing with an undecatrien-3-one (no exact structure given) identified in the *yuzu* fruit (*Citrus junos* Sieb. *ex* Tanaka). This compound was claimed as an ingredient of fresh fruit-like aroma compositions (Miyazawa *et al.* 2009b; Nakanishi und Watanabe 2010; Tomita 2010). Both authors presented (5,7,9)-decatrien-2-one as a fruity flavor (Nakanishi und Watanabe 2010; Tomita 2010). Neither further publications nor spectral data were published so far (SciFinder structural search, Sept. 08, 2019). Reanalysis of fresh pineapple, juices, and canned products *via* solvent extraction techniques as well as stir bar sorptive extraction (SBSE) did not indicate the presence of decatrienones [data not shown; 200 g of pineapple sample; limit of detection (LOD) according to perceived threshold masses shown in Table 3.5.1].

Table 3.5.1. Odor Impressions and Perceived Threshold Masses of (5*E*/*Z*,7*E*,9)-Decatrien-2-one and Structurally Related Compounds

		m_T [ng]	Odor impression
	**10a.b**	5,0	Vanilla, soil like, fruity
	**8a.b**	0,5	Grapefruit, mandarin, orange, fruity, green
	**12**	5,0	Pineapple (fermented), rubber, plastic, bitter, fruity
	**7a.b**	5,0	Pineapple, bitter lemon, flowers, grassy, fruity
	**9b**	23	Citrus fruits, linalool, fruity, green,
	**9a**	18,5	Citrus fruits
	**9c**	0,195	Mandarin, fruity
	**9d**	5,0	Citrus fruits, flowers, fruity
	**6b**	0,028	Pineapple, fresh breath
	**6a**	22	Pineapple, fruity, pungent

[a]m_T = threshold of perceived mass injected on-column and still perceived at the GC sniff port (median of five panellists).

Preparative GC was used for the accumulation of one pure isomer from pooled extracts of the fungal culture medium (as described elsewhere) (Schimanski *et al.* 2013). The purified product was subsequently subjected to NMR analysis. Because both isomers were present after synthesis and further preparative separation was not undertaken, they were measured together. Thus, ^{1}H–NMR gave a double set of signals in the spectrum. For both isomers, the proton at C5 showed ^{3}J couplings with protons at C4

and C6 revealing a multiplet (doublet, triplet), which was confirmed by a 2D proton correlation spectrum (DQF-COSY). In general, coupling constants (^{3}J) of *E* isomers are higher than those of *Z* isomers as a result to the torsion angle of 180°. The multiplet at 5.42 ppm (J = 10.09 Hz) was assigned to the 5(*Z*) isomer and that at 5.69 ppm (J = 14.76 Hz) was assigned to the 5(*E*) isomer. A comparison of the intensity of both signals gave a ratio of 44(*E*)/56(*Z*), the same as was found with GC–MS. The stereochemistry of the double bond at C7 could only be assigned for the 5(*Z*) isomer *via* NMR. The coupling constant (J = 15.0 Hz) reveals a 7(*E*) configuration. Because of overlapping signals for the 5(*E*) isomer, the stereochemistry of the double bond at C7 was not assigned by NMR. According to the synthetic route based on the Wittig reaction, the stereochemistry at the C7 double bond was defined as trans and suggested that the 7(*E*) isomer dominated in the cell cultures. This is explained by an isomerization of the 5(*Z*) isomer to the thermodynamically more stable 5(*E*) form during prolonged incubation at an elevated temperature (25 °C).

Figure 3.5.2 compares mixed ^{1}H–NMR of the synthesized decatrien-2-ones to the spectrum of the pineapple-like impact compound isolated earlier from the culture medium of *F. betulina (Schimanski et al.).* The multiplet at 5.7 ppm, assigned to the proton at C5, in the spectrum of the isolated decatrien-2-one showed that the isomer found in the culture medium of *F. betulina* was unequivocally (5*E*,7*E*,9)-decatrien-2-one.

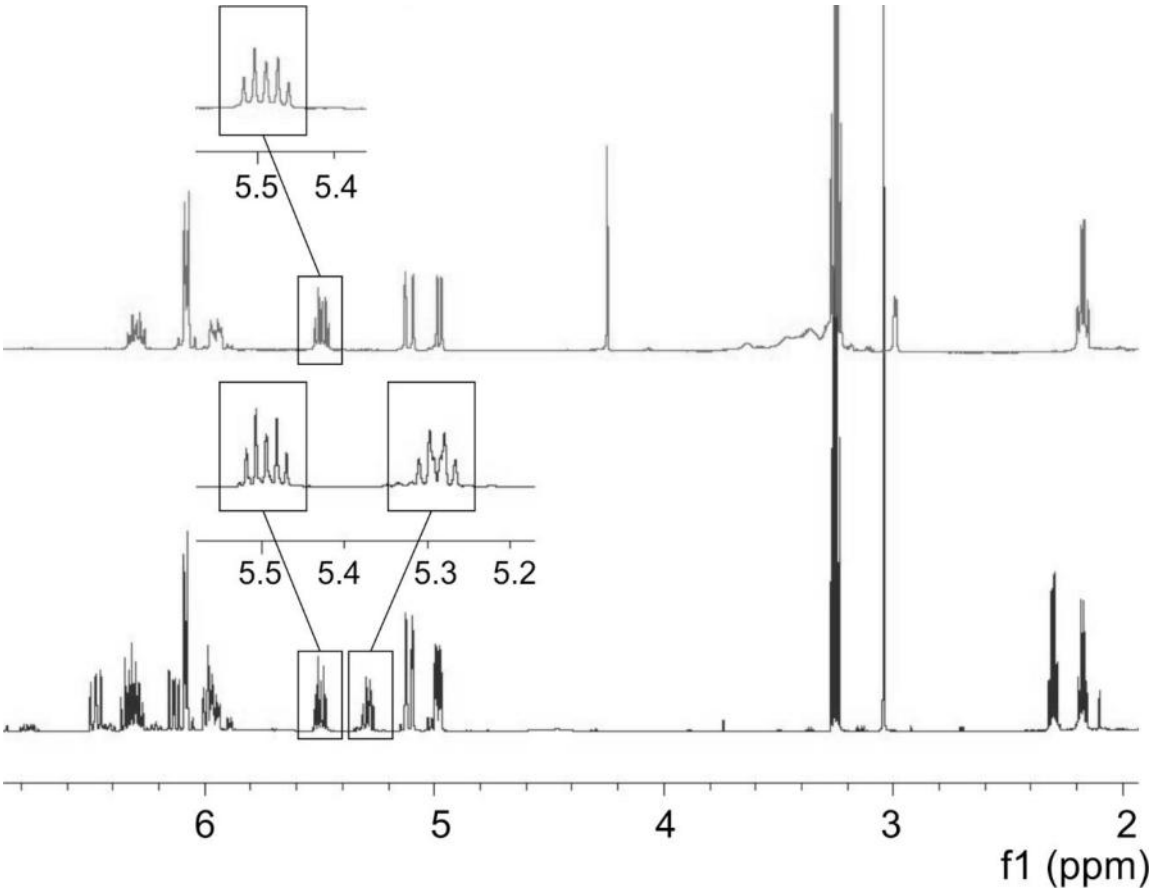


Figure 3.5.2. Comparison of ^{1}H-NMR spectra of the (top) synthesized isomeric mixture of (5*E*/*Z*,7*E*,9)-decatrien-2-one (6a and 6b) and (bottom) isolated (5*E*,7*E*,9)-decatrien-2-one (6a) from *F. betulina*. Signals in frames correspond to the respective proton(s) at C5.

3.5.3 Sensorial Characterization of (5*E*/*Z*,7*E*,9)-Decatrien-2-ones and Structurally Related Compounds

Structurally related methyl ketones were synthesized along the same route. One reactant of the Wittig reaction, protected ketopentanal, was kept constant, whereas the counterpart was varied in chain length as well as number and position of double bonds. The respective structures were confirmed with GC–MS and ^{1}H and ^{13}C–NMR analyses. A sensorial evaluation of all compounds synthesized and commercial 9-decen-2-one was undertaken using GC–O, and perception thresholds were determined by AEDA. If a sufficient chromatographic separation of certain (*E/Z*) isomers was not achieved, the mixed impression of both isomers was recorded (Table 3.5.1). In general, all flavor

compounds were described as fruity. More detailed descriptions resulted in a highly diverse set of attributes. 5,7-Octadien-2-ones (**10a** and **10b**) not only reminded of us vanilla but were also described as soil-like and fruity. Solely, the isomers of (5*E*/*Z*,7*E*)-decatrien-2-one (**6a** and **6b**) were clearly identified as a pineapple aroma by the majority of panelists (4 out of 5). The 5(*Z*) isomer required a 800-fold lower mass for perception (0.028 ng) compared to the 5(*E*)-isomer, with 22 ng. The isomer dominating in the culture medium of *F. betulina* was (5*E*,7*E*,9)-decatrien-2-one with the higher perception threshold mass. However, the relatively low threshold of the *E*-isomer together with its actual concentration was sufficient to provoke the distinct pineapple flavor of the fungal cell culture. Besides, (5,7,9)-decatrien-2-ones further chemosynthesized methyl ketones, such as dec-9-en-2-one and 5,9-decadien-2-ones (**7a** and **7b**), imparting a slight pineapple-like odor impression.

To obtain insight into structural requirements for a pineapple odor impression, a comparison of global energy-minimized conformations (quenched dynamics simulation and DREIDING force field (Mayo *et al.* 1990)) of the respective methyl ketones was performed. The calculated conformations are shown in Figure 3.5.3 and compared to each other and to (1,3*E*,5*Z*)-undecatriene, a long-known genuine character impact compound of fresh pineapple with a low threshold of 7.7 ng/kg of water (Berger und Kollmannsberger). All compounds characterized as typically pineapple-like (**6a** and **6b**; 7**a** and **7b,** and **11**) provided a curved structure consisting of a skeleton of at least 10 carbon atoms with a terminal double bond opposite the methyl ketone terminus. Because no variation of the keto function was performed, its impact was not evaluated directly. However, (1,3*E*,5*Z*)-undecatriene and (1,3*E*,5*Z*,8*Z*)-undecatetraene were described as important constituents of fresh pineapple flavor (Berger und Kollmannsberger; Tokitomo *et al.* 2014). Their odor was described as fruity, pineapple-

like for undecatriene and fresh, citrus-like for undecatetraene (Neiens und Steinhaus 2018). Both olefins do not possess a ketone function but a “L”-shaped conformation, which is more pronounced for (1,3*E*,5*Z*)-undecatriene (Figure 3.5.4). The data suggest that the keto group was not a mandatory structural element to evoke the pineapple sensation. The pineapple-like odor impression was lost, if the length of the carbon chain was shortened (**9a**–**d** and **10a** and **10b**). Although compounds **9b**, **9c,** and 1**0b** show the same “L”-shaped conformation, like (5*Z*,7*E*,9)-decatrien-2-one (**6b**), and additionally, compound **10b** shows the same conjugated pentadienyl system with a terminal double bond, the pineapple character of the flavor was completely lost. Alteration of the double bond system (number, position, and stereochemical orientation) while maintaining the carbon skeleton led to an increase of the thresholds (less intense) for all compounds compared to compound **6b,** but the pineapple impression remains with one exception. Unlike the others, 5-decen-2-one (**8a** and **8b**) lacks a terminal double bond, which seems to be an essential molecular feature to evoke a pineapple-like flavor.

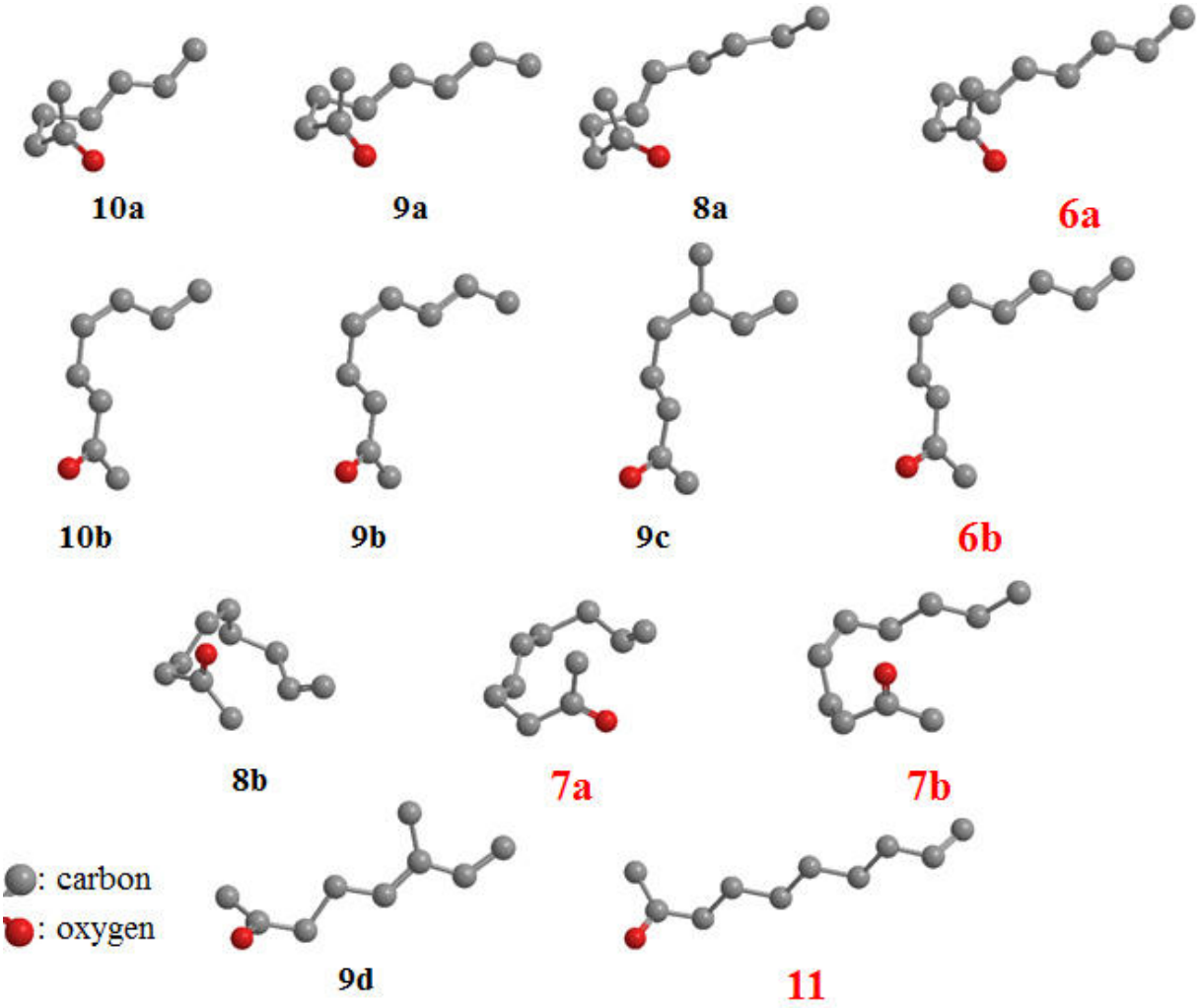


Figure 3.5.3. Energy-minimized structures of methyl ketones prepared (numbering corresponds to Table 3.1). Bold red numbers indicate compounds with a pineapple-like flavor sensation (quenched dynamics simulation and DREIDING force field (Mayo *et al.* 1990)).

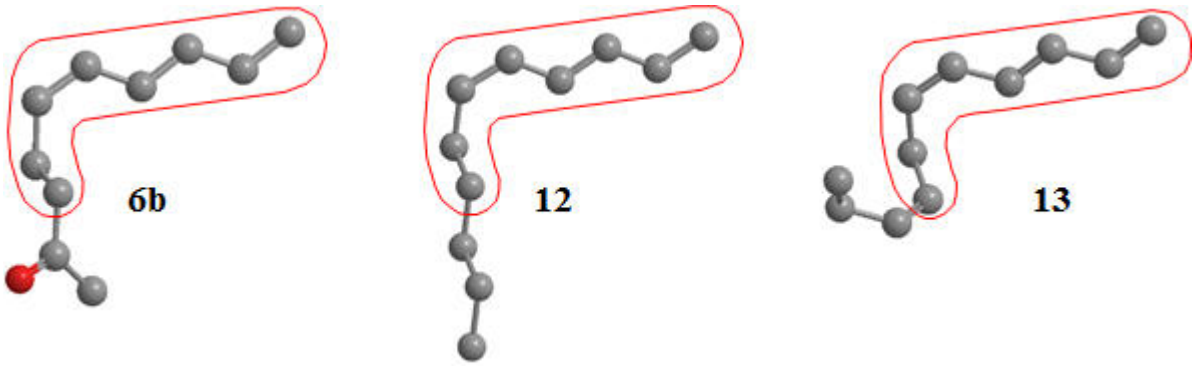


Figure 3.5.4. Comparison of (5*Z*,7*E*,9)-decatrien-2-one (**6b**) to (1,3*E*,5*Z*)-undecatriene (**12**) and (1,3*E*,5*Z*,8*Z*)-undecatetraene (**13**). Energy minimized structures (quenched dynamics simulation and DREIDING force field (Mayo *et al.* 1990)).

For a long time, hexanoic acid prop-2-enyl ester (“allyl caproate”), absent from pineapple volatilome, has been used to imitate pineapple flavor (Ara und Heil 2006; Dravnieks 1985). It shows a nine carbon skeleton, a carbonyl (ester) function and a terminal double bond. Still in use to round off citrus, peach, apricot and apple blossom perfume compositions, it lacks a convincing pineapple note.

3.5.4 Fungal Production of (5*E*/*Z*,7*E*,9)-Decatrien-2-ones

It remains a matter of speculation why a higher fungus should synthesize these compounds. However, in their natural habitats, wood-destructing fungi are exposed to many microbial and animal enemies as well as organisms spreading their spores. Thus, it is not surprising that they interact with their highly populated environment through volatile chemical signals (Orban *et al.* 2019). Like the pineapple constituents (1,3*E*,5*Z*)-undecatriene and (1,3*E*,5*Z*,8*Z*)-undecatetraene, which are known to act as algal pheromones, (5*E*/*Z*,7*E*,9)-decatrien-2-ones with their highly similar three dimensional structure, may serve as signaling compounds in fungi (Boland und Mertes 1985).

The biogenesis of the decatrienones is obscure. Although first detected in cell cultures supplemented with cabbage cuttings in concentrations of 1.1 mg/L, a particular precursor role of this nutrient is doubtful. Fatty acids may serve as a precursor molecule for the formation of methyl ketones along the β-oxidation (Kinderlerer 1993). Fruity constituents of high structural similarity, such as undecatrien-3-one in *yuzu* (*C. junos*), may be formed along this pathway. An analogous origin of the decatrienones of *F. betulina* would require an odd-numbered unsaturated fatty acid, as they in fact occur in basidiomycetes. Supplementing labeled precursors will clarify this question. Another aim of future research could investigate in detail the interaction of decatrienones with human odorant receptor proteins.

4 Formation of Decatrienones with a Pineapple-like Aroma from 1-^{13}C-Acetate by Cell Cultures of the Birch Polypore, *Fomitopsis betulina*

4.1 Vorwort zur Publikation

Nach der Identifizierung von (5*E*/*Z*,7*E*,9)-Decatrien-2-on als neuen Naturstoff wurde dessen Produktion durch *Fomitopsis betulina* charakterisiert. Identifiziert wurde dieser Aromastoff bei der Submerskultivierung auf Weißkohlschnitt als agroindustieller Nebenstrom (Schimanski *et al.* 2013). (5*E*/*Z*,7*E*,9)-Decatrien-2-on weist eine ungewöhnliche Struktur auf. Das geradzahlige Methylketon, welches an den Stellen C5, C7 und C9, jedoch nicht am C3 reduziert ist, ist eine ungewöhnliche Verbindung.

Die Identifizierung der Biogenese in *F. betulina* legt den Grundstein für neue Einblicke in die Maschinerie der Polyketid-Biosynthese. Die Struktur von (5*E*/*Z*,7*E*,9)-Decatrien-2-on ist ungewöhnlich. Die Abwesenheit der Säurefunktion sowie die Ketogruppe ab C2 weisen auf eine ungewöhnliche Polyketid-Synthase mit hin.

Shimin Wu führte die Fettsäureanalytik durch. Ulrich Krings und Ralf G. Berger betreuten diese Arbeit wissenschaftlich und stellten die Projektidee sowie die erforderlichen Mittel.

Bei der nachfolgenden Arbeit handelt es sich um ein akzeptiertes Manuskript, publiziert bei ACS Publications im *Journal of Agricultural and Food Chemistry* am 19.01.2020, verfügbar online unter http://dx.doi.org/10.1021/acs.jafc.9b07494.

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Formation of Decatrienones with a Pineapple-like Aroma from 1-^{13}C-Acetate by Cell Cultures of the Birch Polypore, *Fomitopsis betulina*

Miriam Grosse,* Shimin Wu, Ulrich Krings, and Ralf G. Berger

4.2 Abstract

During submerged cultivation, the edible basidiomycete *Fomitopsis betulina* (previously *Piptoporus betulinus*) developed a fruity odor, strongly reminding of pineapple. Olfactometric analysis showed that this impression was mainly caused by the two (5*E*/*Z*,7*E*,9)-decatrien-2-ones. At the time of maximum concentration on the 5th day, the (5*E*/5*Z*)-ratio was 94:6. Three hypotheses were experimentally examined to shed light onto the genesis of the uncommon volatiles: first, an indirect effect of agro-industrial side-streams, such as cabbage cuttings, supporting good growth; second, an unsaturated odd-numbered fatty acid precursor; third, a polyketide-like pathway. In the presence of 1-^{13}C- or 2-^{13}C-acetate up to five acetates were incorporated into the molecular ions of the C10-body. Addition of 1-^{13}C-pyruvate or 1-^{13}C-lactate did not confirm an odd-numbered starter of the polyketide chain. None of the methylketones was found in pineapple or any other food before.

Keywords: (5*E*/*Z*,7*E*,9)-decatrien-2-one, pineapple odor, polyketide, *Fomitopsis betulina*

4.3 Introduction

Because of its high popularity, pineapple aroma is well researched, and more than 280 volatiles were identified in the fruit (Tokitomo *et al.* 2014). Its rich aroma mainly results from a variety of esters, heterocycles, and polyunsaturated hydrocarbons (Tokitomo *et al.* 2014; Takeoka *et al.;* Berger und Kollmannsberger; Belitz *et al.* 2008; Beauchne *et al.* 2000). A mixture of twelve aroma compounds, including 4-hydroxy-2,5-dimethyl-3(2H)-furanone (pineapple/caramel-like), methyl 2-methylbutanoate (fruity, apple-like), ethyl 2-methylpropanoate and ethyl 2-methylbutanoate (fruity), and (1*E/Z*,3,5)-undecatriene and (1*E/Z*,3,5,8)-undecatetraene (etherical, pineapple-like) in concentrations equal to those of the fresh fruit, resulted in the unique pineapple odor impression (Tokitomo *et al.* 2014). Some of the aroma constituents, *inter alia* 4-hydroxy-2,5-dimethyl-3(2H)-furanone, are common in many fruits and also in heated foods, whereas others, such as (1*E/Z,*3,5)-undecatriene and (1*E/Z,*3,5,8)-undecatetraene rarely occur in food flavors (Berger und Kollmannsberger 1985). No single character impact compound imparting the typical pineapple aroma has become known. Therefore, production of commercial pineapple aroma relies on complex mixtures of flavors compounds. One distinctive natural and typical pineapple-like aroma compound would thus open new opportunities for creating pineapple-flavored products.

The production of natural flavor compounds instead of chemical synthesis is of growing interest. Plants are the major source of natural flavors, but availability and quality are highly influenced by environmental fluctuations. Alternatively, the biotechnological production of natural flavors is a well-controllable and scalable process. Using enzymes or cell factories is a sustainable approach, which may even lead to a cost reduction compared to plant extraction (Krings und Berger 1998). Among the large

variety of microorganisms, higher fungi (*Basidiomycota*) represent a large group of potent aroma producers, either by *de novo* synthesis or by bioconversion (Tkacz und Lange 2004b). For example, *Nidula niveo-tomentosa* produced the key aroma compound of raspberry, 4-(4-hydroxyphenyl)-butan-2-one, along a pathway different from the fruit, *Pycnoporus cinnabarinus* generated vanillin, and *Ceratocystis fimbriata*, a fruity monoterpene mixture reminding of pineapple and banana (Böker *et al.* 2001; Falconnier *et al.* 1994; Christen *et al.* 1997). Schimanski et al. (2013) noted a strong pineapple flavor, which emerged during submerged cultivation of the basidiomycete fungus *Fomitopsis betulina* (*F. betulina*) when grown on industrial side-streams, such as cabbage cuttings (Schimanski *et al.* 2013). The character impact constituents were later on identified as (5*E/Z*,7*E*,9)-decatrien-2-ones with the (5*Z*)-isomer exhibiting an ultra-low threshold of 0.028 ng (Schimanski *et al.* 2013*;* Grosse *et al.* 2019).

The present work describes the time course of formation of this compound in submerged cultures of *F. betulina*, the elucidation of the precursor chemistry using stable isotope labeled substrates, and derives a novel hypothetic acetogenic pathway from the results of the cultivation experiments.

4.4 Materials and Methods

4.4.1 Chemicals and Substances

Media components, chemicals, and solvents were purchased in *p.a.* quality from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). Labeled sodium acetate (1-^{13}C and 2-^{13}C), 1-^{13}C labeled sodium lactate and 1-^{13}C labeled sodium pyruvate were supplied from Sigma Aldrich. All solvents were purified in house by rectification in 1 m silver columns. Extractions were performed with an azeotrope mixture of *n*-pentane and diethyl ether (PE; 1:1.12, *v/v*). The standards of (5*E/Z*,7*E*,9)-decatrien-2 one were synthesized as described elsewhere (Grosse *et al.* 2019).

4.4.2 Cultivation Conditions

Standard nutrient liquid (SNL) at pH 6.0 (30.0 g/L glucose monohydrate, 4.5 g/L l-asparagine monohydrate, 3.0 g/L yeast extract, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 5 µg/L $CuSO_4 \cdot 5\ H_2O$, 80 µg/L $FeCl_3 \cdot 6\ H_2O$, 30 µg/mL $MnSO_4 \cdot H_2O$, 90 µg/mL $ZnSO_4 \cdot 7\ H_2O$, 400 µg/mL EDTA) and minimal medium at pH 6.0 (MM; 3.0 g/L glucose monohydrate, 1.0 g/L yeast extract, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 5 µg/L $CuSO_4 \cdot 5\ H_2O$, 80 µg/L $FeCl_3 \cdot 6\ H_2O$, 30 µg/mL $MnSO_4 \cdot H_2O$, 90 µg/mL $ZnSO_4 \cdot 7\ H_2O$, 400 µg/mL EDTA) were utilized as rich and nutrient-reduced media, respectively, for submerged cultivation. Fungi were maintained on SNL-agar plates containing the medium as described plus 15 g/L agar-agar.

F. betulina [CBS Fungal Biodiversity Centre (Utrecht, the Netherlands), no. 117583] was grown on SNL-agar and maintained at 4 °C. For precultures, 150 mL SNL was inoculated with 1 cm^2 grown agar and homogenized. After 14 days at 150 rpm and 24 °C on a rotary shaker (Infors, Bottmingen, Switzerland), main cultures of 250 mL were inoculated with 25 mL preculture. If not stated otherwise, main cultures were grown in MM with 2% (*w/v,* dry matter) cabbage cuttings (*Brassica oleracea* convar. *capitata* var. *alba*). For monitoring the product formation, *F. betulina* was cultivated in MM with cabbage extracts (see below), SNL without additives and SNL supplemented with 12 mM 1-^{13}C and 2-^{13}C labeled sodium acetate, 2 mM 1-^{13}C labeled sodium lactate or 2 mM 1-^{13}C labeled sodium pyruvate. After 7 days, the main cultures were harvested for analyses.

4.4.3 Isolation of Volatiles

Volatiles were extracted from 250 mL culture supernatant using pentane/ diethylether (PE; 1:1.12 *v/v*). Extracts were dried with sodium sulfate and concentrated by Vigreux

rectification to approximately 1 mL. These extracts were used for qualitative and quantitative analysis.

In time-dependent product analysis, the culture supernatant was extracted by sequential stir bar sorptive extraction using Twisters (10 mm x 0.5 mm, Gerstel, Mülheim, Germany) coated with polydimethylsiloxane (Sampedro *et al.* 2009). This method allows multiple analysis of low concentrated products in small sample volumes. Five milliliter of sample was extracted for 1 h under vigorous stirring at 290 rpm. Afterward, the Twister was rinsed with deionized water, dried with a lint free cloth, and stored at 4 °C. For the second extraction, NaCl was added to a final concentration of 300 g/L and a new Twister was added to the supernatant for another hour. Both stir bars were analyzed together by means of gas chromatography.

4.4.4 Preparation of Lipid Extracts

After 7 days of cultivation the wet mycelia were collected by vacuum filtration of submerged cultures and immediately freeze-dried (Zirbus Vaco 2, Bad Grund, Germany) with a condenser temperature of −49 °C and a chamber pressure of 0.69 mbar for 20-48 h. Dry mycelia were ground to powder and Soxhlet extracted for 4 h (room temperature and protection from light) using pentane/ether (1:1.12, v/v) as the solvent. Extracts were dried over anhydrous sodium sulfate (Carl Roth, Karlsruhe, Germany), concentrated using a Laborota 4000 vacuum rotary evaporator (Heidolph, Schwabach, Germany) with a water bath set at 30 °C, and the remaining solvent evaporated under a gentle stream of nitrogen to constant mass.

Cabbage extracts were prepared using the same procedure. However, fungal extracts were processed further for qualitative analysis, and cabbage extracts were used for main culture supplementation. Five grams of dry cabbage were extracted for each main culture (250 mL).

Prior to analysis, lipid extracts were derivatized with sodium methoxide (Ostermann *et al.* 2014; Liu 1994). In brief, to 100 mg of oil, dissolved in 1 mL of *n*-hexane, 1 mL of sodium methoxide solution (1 % in methanol) was added. The reaction mixture was incubated for 30 min at 50 °C. Afterward, the organic phase was collected, dried, and used for qualitative and semi-quantitative analysis.

4.4.5 Qualitative Analysis

Qualitative analysis of extracts were performed with a 7890B GC-system (Agilent Technologies, Santa Clara, CA, USA) combined with an Agilent 5977A mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionization: 70 eV, scan range *m/z* 33–500 amu; Agilent Technologies). For (5*E/Z*,7*E*,9)-decatrien-2-one identification, two stationary phases of different polarity were installed: DB-WAX MS (30 m, 0.25 mm, 0.25 µm, Agilent J&W GC Columns, Santa Clara, CA, USA) and VF-5 MS (30 m, 0.25 mm, 0.25 µm, Agilent J&W GC Columns). Fatty acid methyl esters were analyzed on a DB-WAX MS column. All analyses were performed with a volumetric flow rate of 1 mL/min helium using following temperature programs: 40 °C for 3 min; 8 °C/min until 230 °C, 230 °C for 10 min (DB-WAX MS) and 40 °C for 3 min, 8 °C/min until 230 °C, 25 °C/min until 325 °C, 325 °C for 10 min (VF 5 MS). Retention indices were calculated using homologue alkanes from C_8 to C_{30}.[14] For the identification of flavor compounds and fatty acid methyl esters, chromatographic data, odor impression (flavor compounds, only), and mass spectra were compared to those of authentic standards (Grosse *et al.* 2019).

Sensory evaluation was performed *via* sniffing culture extracts on an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with a DB-WAX UI column (30 m, 0.32 mm, 0.25 µm; Agilent J&W GC Columns), flame ionization detector (FID) and

olfactory detection port (ODP3, Gerstel, Mülheim, Germany). Separation conditions were chosen as described above.

4.4.6 Quantitative Analysis

Quantitations were performed in a GC 7890B chromatograph (Agilent Technologies) equipped with a FID detector and a polar DB-WAX UI column (30 m, 0.32 mm, 0.25 µm; Agilent J&W GC Columns). For analysis, 1 µL of the sample was injected and separated with hydrogen as carrier gas (2 mL/min) using the following temperature program: 40 °C for 3 min, heating with 10 °C/min until 230 °C, 230 °C for 10 min. Analytes were detected with a FID (250 °C, H_2-flow 35 mL/min, air-flow: 300 mL/min, N_2-flow: 25 mL/min). For all extracts, methyl laurate (40 ng/µL) was used as internal standard. For product quantitation, external calibration using (5*E/Z*,7*E*,9)-decatrien-2-one (1−100 ng/µL) was performed. Lipids were semi-quantified according to the internal standard methyl decanoate (40 ng/µL).

Both stir bars of sequential extraction were measured combined *via* TDS 3-GC-FID (Agilent 6890N, Agilent Technologies) equipped with a cold injection system (CIS 4) (Gerstel, Mülheim, Germany) and FID detector (250 °C, H_2-flow 35 mL/min, air-flow: 300 mL/min, N_2-flow: 25 mL/min). The initial temperature of the TDS 3 was 20 °C. The sample was heated with 60 °C/min to 150 °C and held for 2 min for splitless desorption. Volatiles were re-focused in the CIS 4 at -10 °C on a liner filled with Tenax TA. The CIS was heated to 230 °C with 12 °C/s and held at maximal temperature for 2 min. The sample was injected in the solvent vent mode. As stationary phase, VF-5 MS (30 m, 0.25 mm, 0.25 µm, Agilent Technologies) was used. All analyses were performed with a volumetric flow rate of 1.3 mL/min hydrogen using the temperature program as described above. Quantitations were performed externally using (5*E/Z*,7*E*,9)-

decatrien-2-one (0.015–3 mg/mL) dissolved in methanol. For calibration, 10 µL standard was added to 5 mL of water and proceeded as described below.

4.5 Results and Discussion

4.5.1 Identification of the Compounds Imparting the Pineapple-like Flavor

After 1 day of submerged cultivation, *F. betulina* started to emanate a strong pineapple-like odor. Flavor dilution analysis resulted in the identification of nine key aroma compounds. Besides typical fungal metabolites such as octan-3-one, 1-hexanol, and acetic acid, two compounds with a strong pineapple aroma were identified (Table 4.5.1). GC-MS analysis suggested two stereoisomeric decatrien-2-ones, whereas a related compound was assumed to be a corresponding alcohol. The GC-MS data and retention indices of the biotechnologically produced decatrienones were compared with authentic standards. (5*E*,7*E*,9)-Decatrien-2-one and its (5*Z*)-isomer were well separated on the polar phase. The corresponding alcohol eluted later with an RI of 1956. On the nonpolar column, the (5*E*)-isomer eluted at an RI of 1278 and the (5*Z*)-isomer at 1268, whereas the alcohol eluted at 1294. The mass spectra and retention indices of the biotechnologically generated decatrienones on two stationary phases of different polarity were identical with the synthesized reference compounds. In addition, the odor impressions perceived during GC-O analyses were pineapple-like. Hence, the compounds were identified as (5*E/Z*,7*E*,9)-decatrien-2-ones. In addition to the ketones, the corresponding 5,7,9-decatrien-2-ol was identified.

Table 4.5.1: Key Aroma Compounds of the Culture Supernatant on Day 7 of *F. betulina* Grown on Minimal Medium with Cabbage Cuttings (DB-WAX column)

Compound	**RI[a] [-]**	**RI_{Lit}[b]/Std[c] [-]**	**Odor**
Octan-3-one	1308	1278[b]	Fungal
Hexan-1-ol	1383	1376 [b]	Green
Acetic acid	1454	1460 [b]	Pungent
Benzoic acid methyl ester	1631	1631 [b]	Flowery, herbal
Benzoic acid ethyl ester	1676	1657[b]	Red fruit
(5*Z*,7*E*,9)-Decatrien-2-one	1842	1829[c]	Pineapple
(5*E*,7*E*,9)-Decatrien-2-one	1863	1848 [c]	Pineapple
(5,7,9)-Decatrien-2-ol	1976	1956 [c]	Green
4-Methoxybenzoic acid methyl ester	2105	2092 [b]	Lilac

a: Retention index calculated according to (van den Dool und Kratz 1963). b: Retention index from reference library (National Institute of Standards and Technology). c: Authentic standard

4.5.2 Time Course of the Decatrien-2-one Production

During submerged cultivation, both isomers of (5*E*/*Z*,7*E*,9)-decatrien-2-one were produced. Analysis of the isomer ratio at the point of maximum concentration showed a diastereomeric excess of 88 % for the (5*E*,7*E*,9)-decatrien-2-one in the culture supernatant. However, because the odor threshold of the (5*Z*)-isomer is 800-fold lower (0.028 ng) than that of the (5*E*)-isomer, the strong pineapple odor impression of the culture supernatant is highly influenced by the lower concentrated 5(*Z*)-isomer.(Grosse *et al.* 2019) Because of the low concentrations near the detection limit of the GC-FID method, solely the building kinetics of the (5*E*)-isomer were recorded.

(5*E*,7*E*,9)-Decatrien-2-one was detected from day 2 on (Figure 4.5.1). However, a pineapple-like odor was already perceived on the 1st day indicating an immediate onset of production. The product concentration increased sharply until day 5 to a maximum of 1.1 mg/L. Based on odor thresholds, up to 5.000 L or kg of product could theoretically be flavored with the extract from 1 L culture medium. Concurrently to the methyl ketone, the concentration of 5,7,9-decatrien-2-ol increased, which peaked at 20 µg/L on day 11. This suggested that the ketone was formed first and then slowly reduced in part to the corresponding alcohol. Both product concentrations, ketone and alcohol, rapidly declined within 2 days after passing the maximum concentration. After 14 days of submerged cultivation, neither (5*Z*,7*E*,9)-decatrien-2-one nor decatrienol were detected indicating yet unknown catabolic mechanisms.

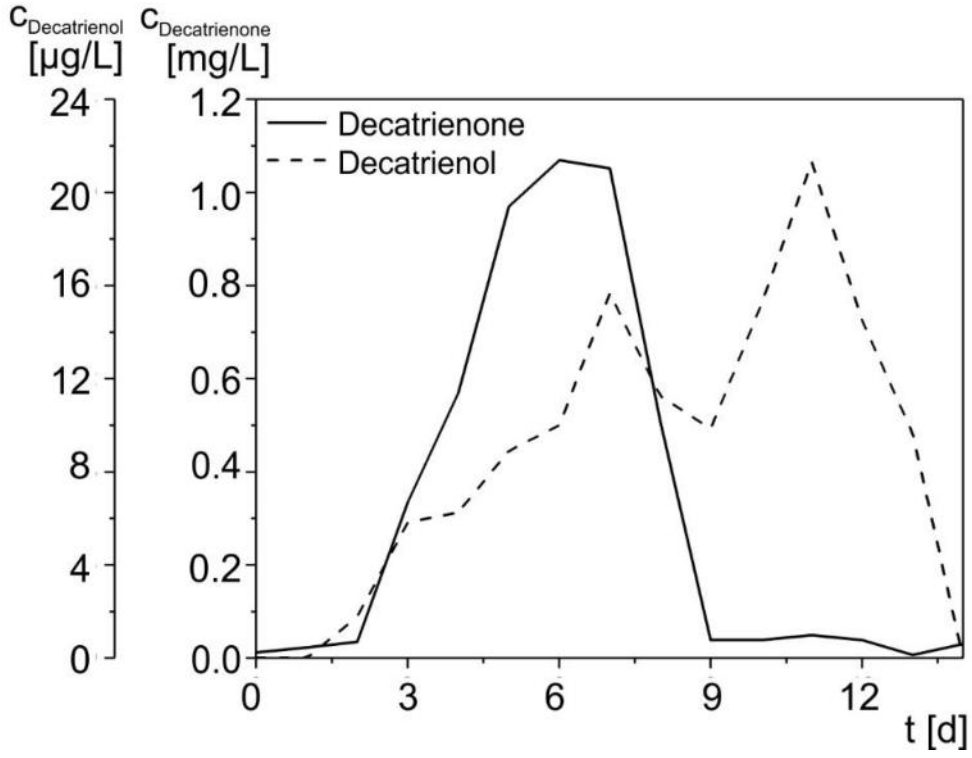

Figure 4.5.1. Time dependent production of (5*E*,7*E*,9)-decatrien-2-one and the corresponding alcohol.

Detailed process analyses in a 5 Lbioreactor showed that the concentration of (5*E*,7*E*,9)-decatrien-2-one rose when the concentration of glucose decreased (see Supporting Information). No direct correlation between fungal growth and product formation was ascertained, but the generation of (5*E*,7*E*,9)-decatrien-2-one obviously depended on good fungal growth. No methyl ketones were produced in culture showing poor mycelial growth. Unlike other secondary fungal metabolites, such as antibiotics, toxins, or signaling compounds, which typically accumulate in the stationary phase, the formation of the decatrienones ceased in senescent cells, clearly indicating an association with primary metabolism.

4.5.3 Characterization of Fungal Biogenesis

Three different hypotheses for the production of the decatrienones were elaborated. The first was an effect of molecules of the cabbage cuttings used as a medium supplement, the second was a catabolic pathway of a suitable odd-numbered fatty acid precursor, and the third was a *de novo* synthesis along a polyketide-like pathway. As volatile linear polyketides were unknown, priority was given to the first two hypotheses.

Decatrienones were first isolated and identified from fungal cultures grown in minimal medium supplemented with remainders from the processing of white cabbage. The vegetable is well known for its mustard oil glycosides, which are rapidly hydrolyzed to a range of bioactive sulfur-containing volatiles, such as nitriles, thiocyanates and isothiocyanates (Belitz *et al.* 2008). Based on the experimental observation, an indirect stimulating effect of these bioactive molecules was a first legitimate guess. However, exchanging the nutrient-limited minimal medium with added cuttings by a rich SNL medium without any cabbage showed a strong increase of the formation of the decatrienones. As only little fungal growth was obtained in minimal medium without

cabbage supplements, it was concluded that the positive effect of cabbage supplementation originated from supporting the active growth of the culture.

Alternatively, the generation of these methyl ketones could be explained along an overflowing β-oxidation of fatty acids (Adda *et al.* 1982; Hawke 1966). The release and hydrolysis of β-keto acetyl coenzyme A (CoA) esters entails a spontaneous decarboxylation to give respective enols which then tautomerize to the corresponding methyl ketones. This process of aroma formation is well known and occurs, for example, in blue cheeses, where ascomycete fungi, such as *Penicillium*, degrade even-numbered fatty acids to odd-numbered methylketones, such as 2-heptanone and 2-nonanone during ripening (Adda *et al.* 1982).

Some rare fruity aroma compounds, such as (6*Z*,8*E*,10)-undecatrien-3-one (yuzunone, Figure 3 B) from the Yuzu fruit, show a similar (ethylketone) structure (Miyazawa *et al.* 2009b). The formation of the even-numbered decatrienones would accordingly require an odd numbered polyunsaturated fatty acid precursor. The fatty acid profile of lipid extracts from *F. betulina* mycelia was analyzed. In accordance with the literature, five odd numbered fatty acids were detected (Table 4.5.2) (Pleszczyńska *et al.* 2017; Grishin *et al.* 2016; Hybelbauerová *et al.* 2008). Heptadecanoic acid was present as odd numbered, monounsaturated fatty acid, but no polyunsaturated odd numbered fatty acids were detected. Literature describes other odd numbered fatty acids, but no polyunsaturated ones among them (Hybelbauerová *et al.* 2008; Reis *et al.* 2011). Decarboxylation of a monoenoic fatty acid would result in less than three double bonds. Thus, none of the unsaturated odd numbered fatty acids of *F. betulina* would result in the desired structure, and decatrienones cannot be expected to result from a genuine fungal fatty acid.

Table 4.5.2: Fatty Acid Profile of *F. betulina* Mycelium (Harvest of Culture Day 7; Fatty Acid Methyl Esters Analyzed on DB-WAX MS)[a]

SFA	c [mg l^{-1}]	MUFA	c [mg l^{-1}]	PUFA	c [mg l^{-1}]
C6:0	0.58±0.13	C6:1	n.d.	C6:2	n.d.
C8:0	tr	C8:1	n.d.	C8:2	n.d.
C12:0	0.70±0.06	C12:1	n.d.	C12:2	n.d.
C14:0	0.51±0.03	C14:1	0.20	C14:2	n.d.
C15:0	0.25±0.01	C15:1	tr	C15:2	n.d.
C16:0	6.92±0.20	C16:1	tr	C16:2	n.d.
C17:0	0.52±0.01	C17:1	0.30±0.02	C17:2	n.d.
C18:0	5.42±0.02	C18:1	37.45±0.16	C18:2	42.93±0.19
		C18:1 (trans)	tr		
C19:0	0.12±0.01	C19:1	n.d.	C19:2	n.d.
C20:0	0.20±0.01	C20:1	n.d.	C20:2	0.45±0.04
				C20:3 (n6)	tr
				C20:3 (n3)	1.07±0.04
C21:0	tr	C21:1	n.d.	C21:2	n.d.
C22:0	0.54±0.02	C22:1	tr	C22:2	n.d.
C23:0	tr	C23:1	n.d.	C23:2	n.d.
C24:0	1.81±0.12	C24:1	tr	C24:2	n.d.

[a] SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; tr: traces; n.d.: not detected

4.5.4 Decatrienones are Most Likely Volatile Polyketide Metabolites

The only option remaining was a *de novo* synthesis *via* a polyketide-like pathway. To verify this, rich SNL medium was supplemented with 1-^{13}C or 2-^{13}C labeled sodium acetate. Presuming that a surplus of acetate would trigger the polyketide pathway, the formation of at least weakly ^{13}C-labelled decatrienones was expected.

The mass spectrum of (5*E/Z*,7*E*,9)-decatrien-2-one obtained from the cultivation with labeled acetate is shown in Figure 4.5.2. Unlabeled C10-compounds showed one natural ^{13}C isotope peak of 11 % intensity of the molecular ion (*m/z* = 150) (McLafferty und Tureček 1995). Compared to this, the intensity of the ^{13}C isotope peak of the decatrienone originating from the labeling experiment (*m/z* = 151) was increased to 19.5 %. Four additional new signals appeared at *m/z* 152, 153, 154, and 155, indicating incorporation of up to five acetate units into the C10-body. Mass spectra looked similar regardless of whether 1-^{13}C or 2-^{13}C acetate was supplemented. It was concluded that (5*E/Z*,7*E*,9)-decatrien-2-one is an unusually short linear methyl ketone generated *via* the polyketide pathway. Conversely, it is highly unlikely that up to five labeled acetates were incorporated consecutively into a growing fatty acid chain and then, after several desaturation reactions, degraded to yield just this C10-body.

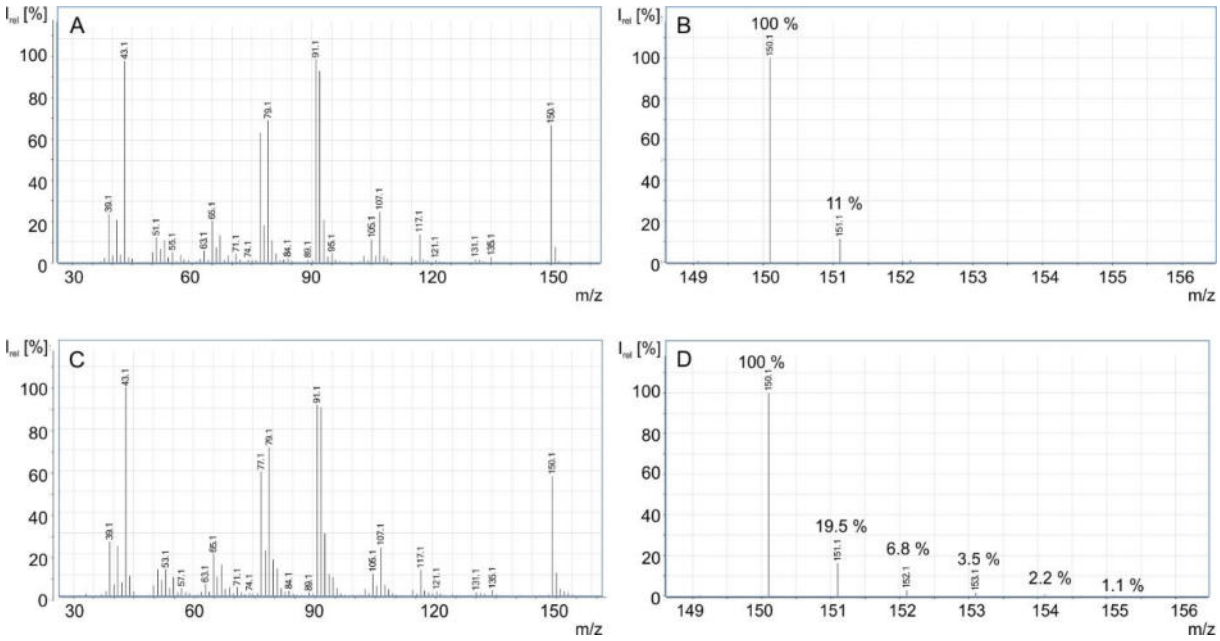

Figure 4.5.2. Mass spectra of (5*E*,7*E*,9)-decatrien-2-ones. (A) Spectrum of unlabeled (5*E*,7*E*,9)-decatrien-2-one (scan m/z from 33 to 300). (B) Intensities of the (5*E*,7*E*,9)-decatrien-2-one molecular ion and isotopic peaks of unlabeled compound (scan m/z from 140 to 160). (C) Spectrum of labeled (5*E*,7*E*,9)-decatrien-2-one (scan m/z from 33 to 300). (D) Intensities of the (5*E*,7*E*,9)-decatrien-2-one molecular ion and isotopic peaks of the labeled compounds (scan m/z from 140 to 160).

For the regular polyketide biosynthesis of an even numbered polyene, an unusual, odd numbered starting molecule would be needed to form an even numbered methyl ketone. Pyruvyl-CoA as well as lactoyl-CoA served as such in a previous study (He *et al.* 2014). Chain elongation would be realized by malonyl-CoA derived from acetate until a C11-body is formed. After hydrolysis of the acyl-CoA intermediate, the terminal double bond would have to be formed *via* a concluding decarboxylation. However, supplementing the presumed starting molecules did not result in labeled compounds (Table 4.5.3).

Table 4.5.3. Intensities of the (5*E*,7*E*,9)-Decatrien-2-one molecular ion (150) and isotopic peaks (151 and 152) in the supernatant after 5 days of main culture of SNL supplemented with 1-^{13}C-lactate, 1-^{13}C-pyruvate and without supplements (SNL control)[a]

m/z	**1-^{13}C-lactate**	**1-^{13}C-pyruvate**	**SNL-control**
150	100	100	100
151	11	11	11
152	0	0	0

[a] scan *m/z* from 33 to 300.

To add some evidence to the polyketide hypothesis, the spectrum of known metabolites of Polyporales, such as *F. betulina* (formerly named *Piptoporus betulinus),* was examined for related polyene structures. *Piptoporus australiensis*, a close relative of *F. betulina*, was the first basidiomycete described as a producer of piptoporic acid (Gill 1982). Schwenk et *al*. (2014) investigated the fungal polyene biogenesis and identified piptoporic acid as a product of polyketide synthesis (Schwenk *et al.* 2014). Laetiporic acid from the edible *Laetiporus sulfureus*, another member of the order Polyporales, and other fungal polyene metabolites were recently shown to involve the polyketide pathway and a double bond shifting activity (Herz *et al.* 1987). Structural

similarities of the decatrienones and substructures of the mentioned polyunsaturated pigments are obvious (Figure 4.5.3). Thus, the synthesis of the decatrienones may result from an interrupted chain elongation during pigment production. Unlike the decatrienones, piptoporic as well as laetiporic acid show a methyl branch next to the carbonyl group. This methyl group is solely introduced in methionine rich media and takes place after the linear chain was formed, as was shown using ^{13}C-labeled methionine (Schwenk *et al.* 2014). Thus, it is concluded that *F. betulina* produced this unusually short polyketide along with larger polyketides.

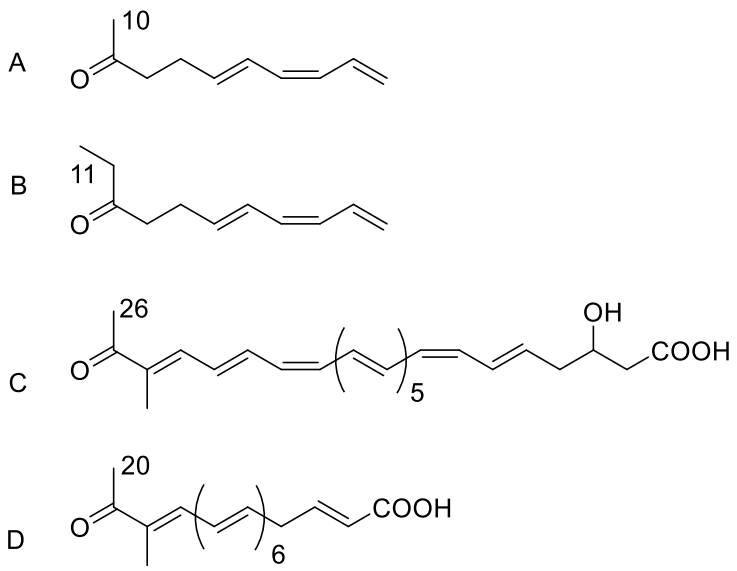

Figure 4.5.3. Structures of (A) decatrienone, (B) yuzunone, (C) laetiporic acid (from *Laetiporus sulphureus*), (D) piptoporic acid (from *Piptoporus australiensis*), polyketide polyenes produced by fungi of the order Polyporales.

The short and even numbered (5*E/Z*,7*E*,9)-decatrien-2-ones are exceptional structures. Either an odd numbered precursor, which is later decarboxylated, or chain formation and elongation by acetyl-CoA units followed by an extensive modification of the resulting ketocarboxylic acid could be envisaged. Further characterization of the polyketide product spectrum of *F. betulina* will follow. This will focus on the nonvolatile

fraction of *F. betulina* and the polyketide multienzyme cluster to elucidate the full polyene spectrum, thereby enhancing our understanding of this uncommon pathway.

4.6 Supporting information

4.6.1 Bioreactor Cultivation

Experimental setup. For complete process analytics of a decatrienone production process, *F. betulina* was cultivated in a 5 L Minifors 2 bioreactor (Infors HT, Switzerland) equipped with a oxygen probe VisiFerm (Hamilton, Massachusetts, USA), a pH probe EasyFerm (Hamilton, Massachusetts, USA) and off gas analysis BlueInOne Cell (BlueSens gas sensor GmbH, Germany). The reactor was operated with 3 L. As main culture medium minimal medium supplemented with 2 % cabbage cuttings (see Materials and methods - Cultivation conditions) was used. The fermenter was inoculated with 400 mL pre-culture grown in SNL for 14 days. During inoculation the bioreactor was aerated with 0.2 vvm (0.8 L/min) and 120 rpm. During cultivation pH was controlled with 2 M NaOH and 2 M acetic acid at pH 6.0 and the pO_2-value at 20 % (probe was calibrated with 100 % air) *via* increasing aeration (F_G). Twice a day, samples of 10 mL were taken for offline analysis. These samples were used to analyze the glucose concentration and the decatrienone concentration.

Glucose concentrations were analyzed according to the GOPOD assay (Megazyme, Irishtown, Ireland). Therefore 10 µL sample were mixed with 290 µL reagent. Glucose oxidase turned D-glucose to D-gluconate and hydrogen peroxide. The second product is used by a peroxidase to produce a quinone imine dye, which was detected at 510 nm. External standards of D-glucose (0.2 – 1.0 g/L) were used for calibration.

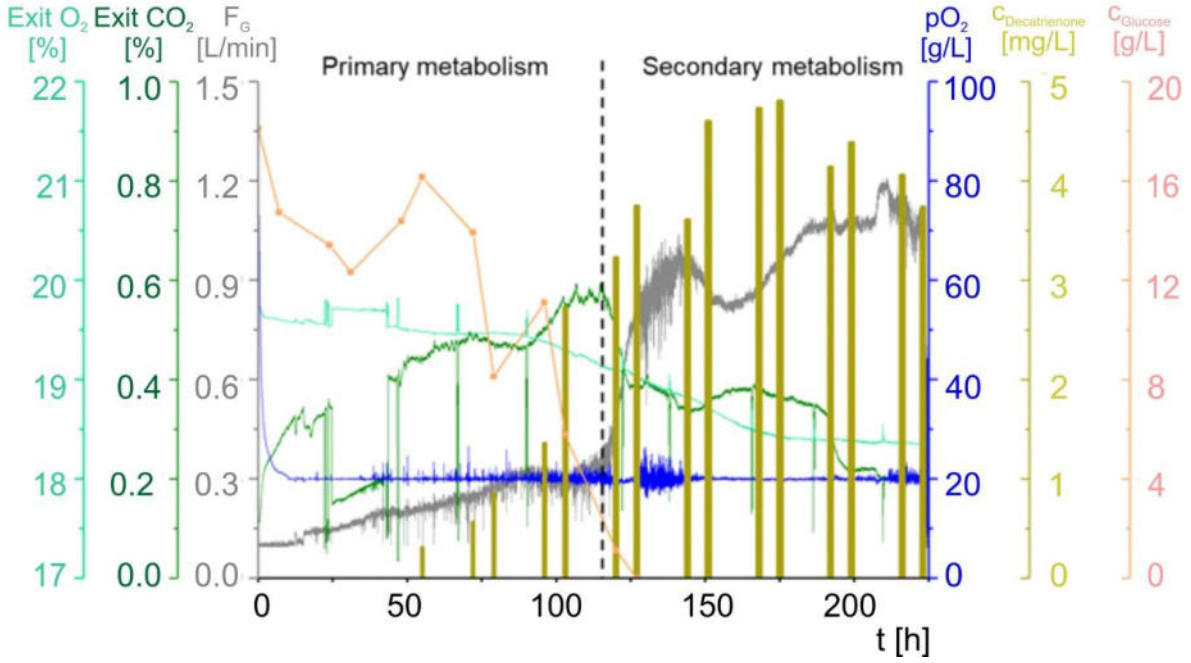

Figure 4.6.1. Bioreactor process of *F. betulina* for (5*E*,7*E*,9)-decatrien-2-one. Process performed in a Minifors 2 (Infors HT, Switzerland) with 3 L working volume (24°C; 120 rpm, pH 6.0) with a controlled oxygen level of 20 %.

Full process characterization. Immediately after inoculation the pO_2 value declined rapidly within 10 h. When the value of 20 % was reached, the aeration was increased in a linear profile for the following 120 h. In parallel, the exhaust CO_2 increased, and glucose was consumed. After 52 h of cultivation the glucose concentration dropped rapidly, and (5*E*,7*E*,9)-decatrien-2-one production was initiated. Product concentration rapidly increased for the following 100 h, whereas glucose concentration declined. A metabolic change of fungal cultures was observed after 120 h, when the exhaust CO_2 peaked and the slope of aeration increased rapidly to achieve the desired pO_2 of 20 %. It was concluded that this point indicated the change from primary to secondary metabolism. After 130 h, the glucose was fully metabolized, and (5*E*,7*E*,9)-decatrien-2-one concentration remained constant at a maximum of 5.5 mg/L. After 175 h the product concentration started to decline. As no further carbon source was available in

the medium (130 h), the product formation via the polyketide pathway was terminated.

Hence, a correlation of fungal growth and production was concluded.

4.6.2 Yuzunone Biogenesis

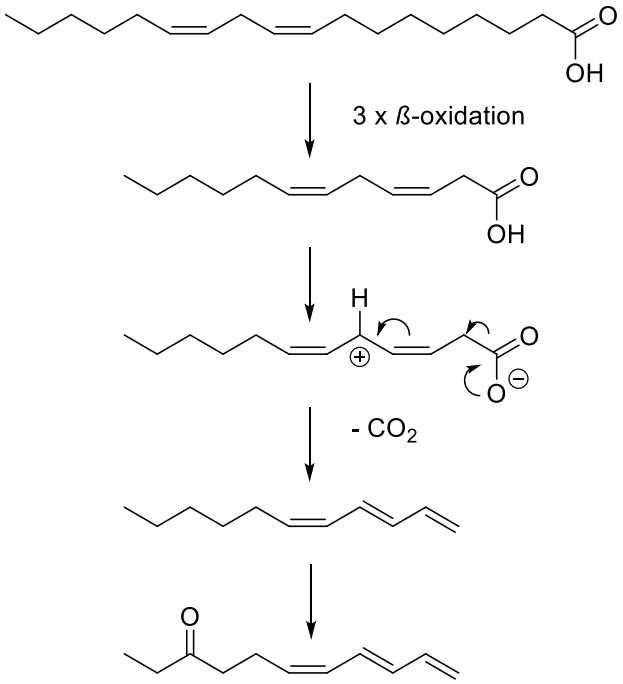

Figure 4.6.2. Hypothetical yuzunone biosynthesis. The β-oxidation of the polyunsaturated fatty acid linoleic acid followed by a decarboxylation may result in the desired ethyl ketone (6*Z*,8*E*,10)-undecatrien-3-one. Experimental proof is still missing.

5 Response of the sesquiterpene synthesis in submerged cultures of the Basidiomycete *Tyromyces floriformis* to the medium composition

5.1 Vorwort zur Publikation

Wie in Kapitel 1.3.3 dargelegt, wird bei der Sesquiterpenproduktion von Basidiomyceten eine komplexe Interaktion zwischen Pilz, Pflanze und Insekten vermutet (Kramer und Abraham 2012). Diese Hypothese basiert auf zahlreichen Einzelbeispielen. Systematische Untersuchungen zu diesem Thema fehlen. *Tyromyces floriformis* wurde in institutsinternen Arbeiten, sowie in der Literatur als potenter Sesquiterpenproduzent identifiziert und deshalb als Modellorganismus für diese verwendet. Im Rahmen eines breiten Screenings wurden biotische und abiotische Effektoren identifiziert.

Die vorliegende Arbeit bietet Einblicke in die Regulation der Sesquiterpen-Biosynthese von Pilzen. Ergebnisse zeigen, dass in der Kultur beobachtete Effekte klar auf Schlüsselenzyme zurückgeführt werden können. Diese Erkenntnisse legen den Grundstein für weitere systematische Charakterisierungen besonders auf genomischer Ebene.

Elisa Strauss führte im Rahmen einer Bachelorarbeit die Kultivierungen der Übertragbarkeitsstudie vom Modellorganismus *Tyromyces floriformis* auf die Basidiomyceten *Cerrena unicolor*, *Postia placenta* sowie *Coprinopsis cinerea* durch. Ulrich Krings sowie Ralf G. Berger übernahmen die wissenschaftliche Betreuung sowie die Bereitstellung des Themas. Weiterhin stellte Herr Berger die nötigen Mittel für die Forschungsarbeiten zur Verfügung.

Bei der nachfolgenden Arbeit handelt es sich um ein akzeptiertes Manuskript publiziert bei Taylor & Francis im Journal *Mycologia* am 22.10.2019, verfügbar online unter https://doi.org/10.1080/00275514.2019.1668740.

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Response of the sesquiterpene synthesis in submerged cultures of the Basidiomycete *Tyromyces floriformis* to the medium composition

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5.2 Abstract

Tyromyces floriformis, a potent fungal sesquiterpene producer, was grown[*1] as a model organism in submerged culture to search for chemicals affecting sesquiterpene biosynthesis *in vitro*. Thirty-one sesquiterpenes and sesquiterpenoids were identified in the supernatant, among them the fruity *α*-ylangene as main volatile. Additives, such as some polysaccharides or lipids, did not affect the qualitative product spectrum but strongly affected the quantitative synthesis. Rye arabinoxylan and other polysaccharides, such as chitin, starch, and agarose almost blocked the synthesis of *α*-ylangene. Single addition of the building blocks of arabinoxylan, arabinose, xylose, or ferulic acid, showed no inhibitory effect, whereas 0.05 % (*w/v*) 3^2-*α*-l-Arabinofuranosyl-xylobiose and larger oligosaccharides resulted in a significant suppression. In contrast, addition of acetyl donors boosted the *α*-ylangene concentration by 1 order of magnitude up to > 40 mg L^{-1}. Both, increased as well as decreased *α*-ylangene concentrations correlated with the intracellular sesquiterpene cyclase activity. Similar experiments using submerged cultured *Cerrena unicolor*, *Postia placenta* and *Coprinopsis cinerea* showed that the additives affected fungal sesquiterpenoid synthesis differently. Whereas the addition of acetyl donors boosted the synthesis in all biphasic cultures, it was inhibited by polysaccharides in fungi preferably interacting with lignified plants. In contrast, *Cerrena unicolor*, known for a symbiotic lifestyle with wasps, responded by forming higher concentrations of the possibly insect-attracting sesquiterpenes.

[*1] Change on behalf of the correction note made on the 22nd of January 2020.

Keywords: *Tyromyces floriformis*; Sesquiterpene synthesis; α-Ylangene; Arabinoxylan; Tegosoft; Volatilome

5.3 Introduction

The turnover of flavors accounted for 24.8 billion US $ in 2017 and for more than a quarter of the worldwide food additives market (Ben Akacha und Gargouri 2015; International Flavors & Fragrances Inc. 2018). Natural flavors, obtained from biotechnology, satisfy customers' demands on product quality and safety. Among them, terpenoids present the largest group with the highest structural variety (Stevens 1992; Bohlmann 1984). Although terpene hydrocarbons tend to autoxidize and often impart unspectacular scents, oxo-functionalized mono- and sesquiterpenoids like the grapefruit-like smelling (+)-nootkatone often impart strong odor impressions (Leonhardt und Berger 2015). However, sesquiterpenes are useful as aroma precursors (Ohloff *et al.* 2012), and their sustainable biotechnological production is of interest for both aroma chemistry and as synthones for pharmaceuticals (Schwab *et al.* 2013). The fruity-smelling *α*-ylangene is one among the aroma-active sesquiterpene hydrocarbons. It is a major component of ylang ylang (*Cananga odorata*) essential oil (Jin *et al.* 2015). Due to its pleasant scent, ylang ylang oil is an appreciated ingredient in perfumes, shampoos, and soaps. The most popular product is Chanel N° 5, which contains up to 10% ylang ylang oil (Coconuts Manila 2015). In addition, *α*-ylangene influences the peppery aroma of *Shiraz* grapes and was identified in various fruits and spices, for example in *Schisandra chinensis* fruits and cinnamon, and also in cloves (Jurado *et al.* 2007; Siebert *et al.* 2008; Chen *et al.* 2012).

Unlike chemosynthesis and plant extraction, the biotechnological production of flavors results in natural products (regulation EC 1334/2008) of reliable quality and yield. First reports on volatile fungal terpenes go back to 1963 (Sprecher 1963). Higher fungi, especially Basidiomycota, are potent aroma producers. Their spectra of aliphatics, aromatics, and terpenoids are often close to plant volatile profiles (Abraham und

Berger 1994). Nowadays, more than 300 sesquiterpene scaffolds are known (Miller und Allemann 2012).

The mevalonate pathway of fungal sesquiterpene biosynthesis starts with isopentyl and dimethylallyl pyrophosphate building blocks (IPP and DMAPP) derived from acetyl-coenzyme A (acetyl-CoA). The characteristic C15 backbone of sesquiterpenes is composed of three of them, forming the linear farnesyl diphosphate (FPP). Specific sesquiterpenes are formed by a Type I sesquiterpene synthases by binding the substrate FPP and liberating the pyrophosphate group (Lesburg 1997; Starks *et al.* 1997; Cane *et al.* 1995). The resulting reactive carbocation is stabilized in the active center of the enzyme. Products are formed by rearrangement, cyclization, hydride shifts, and formation of new C-C bonds (Miller und Allemann 2012). The predominant products of Basidiomycota are derived from 1,6- and 1,10-cyclization, such as germacrene, bisabolene, cadinene, and drimane (Fraga 2013; Zheng *et al.* 2013; Jansen und Groot 2004). Several sesquiterpenes may be produced by the same cyclase (Steele *et al.* 1998; Pichersky *et al.* 2006).

Considered to be a kind of metabolic waste or energy depot until the 1970s, the ubiquitous sesquiterpenes are nowadays recognized for their essential physiological functions, *inter alia* as intra- and interspecies signaling molecules (Gershenzon und Dudareva 2007). Recent research revealed a well-balanced and complex fungi-plant-insect interaction (Kramer und Abraham 2012). The synthesis of sesquiterpenes was shown to be influenced by the cultivation conditions (Freihorst *et al.* 2018). However, more systematic investigations are missing.

The present work shows how the sesquiterpene synthesis of fungi may be affected *in vitro* by chemicals occurring in their natural habitat. First, the sesquiterpene product

spectrum of the model organism *Tyromyces floriformis* (*T. floriformis*) was characterized. Different compounds were added to the culture medium to address suspected fungi-plant-insect interactions, to refill the acetate pool, and to supply facultative precursors of terpene synthesis. Furthermore, the effects were correlated with the activity of the key enzyme involved, and finally the experiments were extended to some other sesquiterpene producing basidiomycete species.

5.4 Materials and Methods

5.4.1 Chemicals and substances

All media components, chemicals, and solvents were purchased, if not mentioned otherwise, in *p.a.* quality from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). Rye arabinoxylan, 3^2-*α*-L-arabinofuranosyl-xylobiose (A^3X), 2^3,3^3-di-*α*-L-arabinofuranosyl-xylotriose ($A^{2+3}XX$) and 3^3-*α*-L-arabinofuranosyl-xylotetraose (XA^3XX) were from Megazyme (Bray, Ireland). Tegosoft (PEG-7 Glyceryl Cocoate, an emulsifier used in cosmetics) was purchased from Evonik Industries (Essen, Germany), castor oil from Henry Lamotte Oils (Bremen, Germany), and rapeseed oil from Rapso (Aschach, Austria). Farnesyl pyrophosphate ammonium salt was obtained from Sigma Aldrich (Steinheim, Germany). For solvent extractions, an azeotropic mixture of *n*-pentane and diethyl ether (1/1.12, *n/n*) was used. All solvents were purified by rectification before use.

5.4.2 Cultivation conditions

Submerged cultivations were performed in standard nutrient liquid at pH 6.0 (SNL; 30.0 g L^{-1} glucose monohydrate, 4.5 g L^{-1} L-asparagine monohydrate, 3.0 g L^{-1} yeast extract, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 5 µg L^{-1} $CuSO_4 \cdot 5\ H_2O$, 80 µg L^{-1} $FeCl_3 \cdot 6\ H_2O$, 30 µg mL^{-1} $MnSO_4 \cdot H_2O$, 90 µg mL^{-1} $ZnSO_4 \cdot 7\ H_2O$, 400 µg mL^{-1} EDTA).

Fungi were maintained on SNL-agar plates containing the medium as described plus 15 g L^{-1} agar-agar.

Fungal species were purchased from commercial culture collections or were laboratory isolates: *Tyromyces floriformis* (CBS Fungal Biodiversity Centre, Utrecht, Netherlands, No. 232.53), *Cerrena unicolor* (CBS Fungal Biodiversity Centre, Utrecht, Netherlands, No. 154.29), *Postia placenta* (US Department of Agriculture, No. Mad-698-R), and *Coprinopsis cinerea* (self-isolate, identification confirmed by ITS sequencing).

Fungi were grown on SNL-agar and maintained at 4 °C. Pre-cultures were grown in shake flasks of 250 mL SNL for seven days at 150 rpm and 24 °C on a rotary shaker (Infors, Bottmingen, Switzerland). For inoculation of the precultures, 1 cm^2 grown SNL-agar of the respective fungi was transferred into a separate preculture flask and homogenized before incubation. Main cultures were grown in SNL medium with or without additives. Additives were selected based on preliminary experiments with *T. floriformis* on complex agro industrial side streams (data not shown). Polysaccharide additives of 0.05 % *(w/v)* starch from potatoes, sodium alginate, chitin, agarose, and rye arabinoxylan were used. Additional feeding experiments with 0.05 % *(w/v)* each of fractionated and nonfractionated rye arabinoxylan, A^3X, $A^{2+3}XX$, XA^3XX, as well as the arabinoxylan building blocks, namely ferulic acid, arabinose, and xylose were performed. Lipidic supplements of up to 6 % *(v/v)* Tegosoft, rapeseed, castor, and paraffinic oil as well as linoleic acid were investigated. SNL medium without additives was the reference.

All main cultures were inoculated with 1/10 (*v/v*) of preculture. Cultures supplemented with A^3X and $A^{2+3}XX$ were grown on the 50 mL scale. The scalability of product concentrations of different culture volumes using 250 mL, 200 mL, 100 mL, 50 mL, and

25 mL were compared. After 21 days, cultures were harvested, and the supernatant was analyzed.

5.4.3 Characterization of shaking flask cultivations

The pH and pO_2-values were monitored online with the shake flask reader SFR vario (PreSens, Regensburg, Germany). In the shaking flask, sensitive patches were used to report online values of dissolved oxygen and pH (Figure 5.4.1). Subsequent sampling was done for glucose and volatilome analyses. D-Glucose was measured offline using a glucose oxidase/peroxidase assay (GOPOD-format) by Megazyme (Bray, Ireland) according to the manufacturer´s protocol. External calibration with 0.05-1.0 mg mL^{-1} D-glucose was performed.

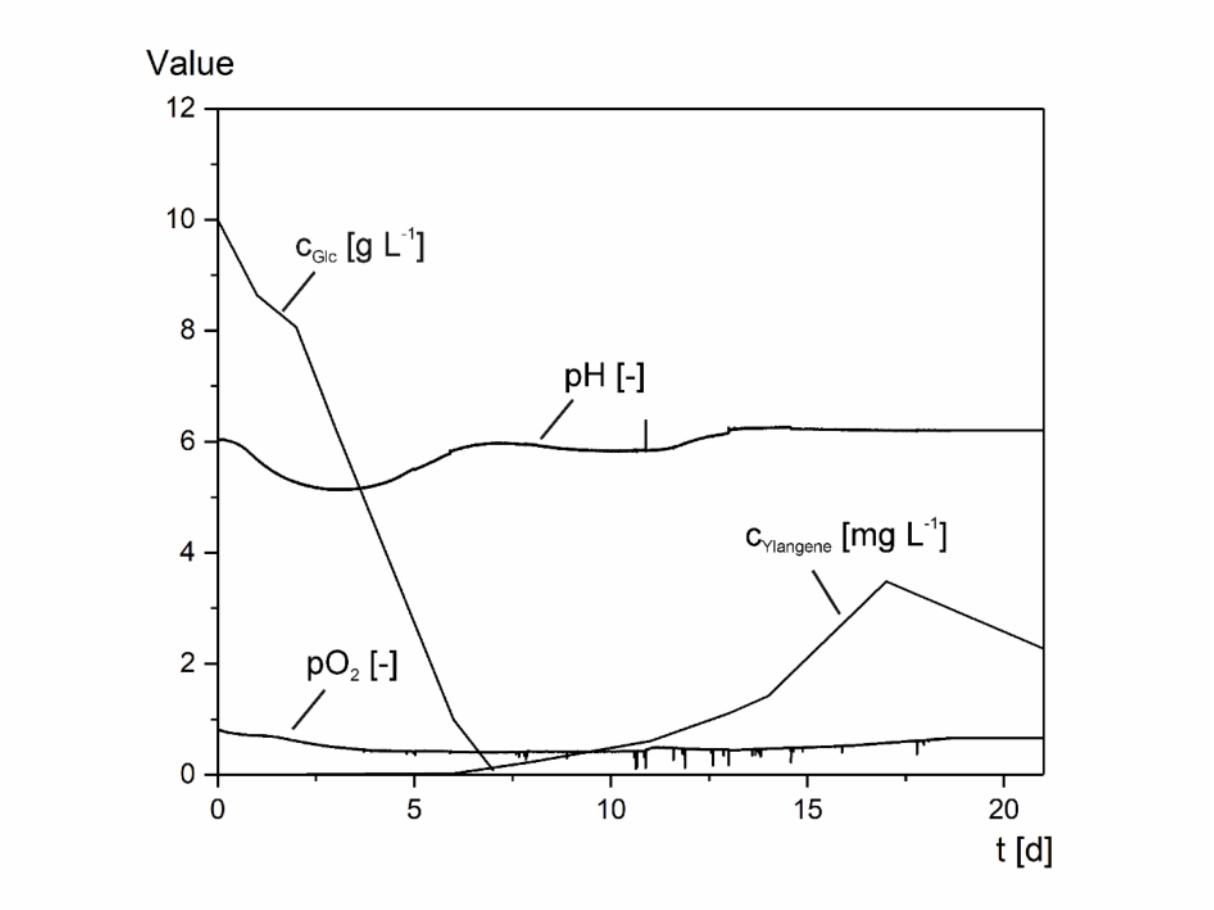

Figure 5.4.1. Representative course of cultivation parameters: shake flask cultivation of *T. floriformis* (SNL medium, 250 ml, non-baffled shake flask, 24 °C, 150 rpm, 21 days).

5.4.4 Volatilome analysis

Volatiles were extracted from the culture supernatant by sequential stir bar sorptive extraction (SBSE) using Twister (10 mm x 0.5 mm, Gerstel, Mülheim, Germany) coated with polydimethylsiloxane (PDMS) (Sampedro *et al.* 2009). This method facilitates solvent free product accumulation with few losses, especially for low-concentrated complex samples (Trapp *et al.* 2018). Due to large bed volumes, SBSE provides higher recovery rates and less competition among different analytes than solid-phase microextraction (SPME) (Barba *et al.* 2017). Therefore, a more representative extract of the culture supernatant is produced. Extractions were performed according to the sequential Twister extraction. This method favors the uniform and quantitative extraction of a wide product range with different polarities (Marsili 2016; Jeleń *et al.* 2012).Five milliliters culture supernatant was extracted for one hour. Afterwards, the solution was brought to 30 % (*w/v*) sodium chloride for an extraction using a second Twister. After another hour, the second stir bar was removed from the sample. Both stir bars were rinsed after accumulation with pure water and dried with a lintfree cloth. Until measurement, the Twister were stored in vials at 4 °C.

As lipid rich culture supernatants cannot be analyzed by SBSE, liquid-liquid extraction using 50 mL *n*-pentane/diethyl ether was performed. The extraction was repeated twice. Organic extracts were pooled, dried with sodium sulfate, and concentrated using Vigreux rectification (DURAN, Wertheim, Germany) at 43 °C. Analysis *via* thermodesorption-gas chromatography-mass spectrometry and olfactometry (TDS-GC-MS/O) was performed as described elsewhere (Schimanski *et al.* 2013).

Qualitative and quantitative analyses of volatiles were performed with an Agilent 6890N chromatographic system (Agilent Technologies, Santa Clara, USA) in

combination with the Agilent 5975B mass-selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionization: 70 eV, scan range *m/z* 33–500 amu). As stationary phases DB-WAX MS (30 m, 0.25 mm, 0.25 µm, Agilent Technologies) and VF-5 MS (30 m, 0.25 mm, 0.25 µm, Agilent Technologies) were used. All analyses were performed with a volumetric flow rate of 1.3 mL min^{-1} helium using the following temperature programs: 40 °C hold for 3 min; 5 °C min^{-1} until 230 °C, 230 °C hold for 10 min (DB-WAX MS) and 40 °C hold for 3 min, 5 °C min^{-1} until 230 °C, 25 °C min^{-1} until 325 °C, 325 °C hold for 10 min (VF 5 MS).

Samples were injected with a thermal desorption system (TDS 3) and a cold injection system (CIS 4) (Gerstel, Mülheim, Germany). The initial temperature of the TDS 3 was 20 °C. The sample was heated with 60 °C min^{-1} to 150 °C and held for 2 min for desorption in the splitless mode. Volatiles were refocused on a liner in the CIS 4 filled with Tenax TA at −10 °C. Afterwards, the CIS was heated to 230 °C with 12 °C s^{-1} and held for 2 min in the solvent vent mode.

Sesquiterpenoids were identified by their respective retention indices according to van den Dool and Kratz using *n*-alkanes (C_8 – C_{25}) as external references, reference materials and database search with the commercial database NIST 14 (van den Dool und Kratz 1963). For the semiquantification of lipid free samples, external calibration with *α*-bisabolol was carried out. This method allowed the quantification of low concentrations. Lipid samples were semi-quantified using *α*-bisabolol (100 µg mL^{-1}) as internal standard.

5.4.5 Fractionation of Arabinoxylan

To understand the effect of the chain length of arabinoxylans on the *α*-ylangene synthesis, the polycondensate was fractionated using high performance liquid

chromatography (HPLC). Rye arabinoxylan was dissolved in water (10 mg ml^{-1}) and autoclaved to create a concentration similar to the culture broth. The solution was centrifuged (5 min, 7500×g, Hettich, Rotina 460 R, Tuttlingen, Germany) and filtrated (syringe filter Chromafil® RC-45/25, Macherey-Nagel, Düren, Germany). Fractionation was performed with the Hitacho Chromaster (VWR, Radnor, Pennsylvania) using a Waters Ultrahydrogel 1000 column (Waters Corp., Milford, Massachusetts), La Chrom RI detector L7490 (Merck, Darmstadt, Germany) and a BioFrac fraction collector (Biorad, Hercules, California). As mobile phase, 0.6 mL min^{-1} degassed water was used. Fifty microliter arabinoxylan solution were injected for each run. Fractions were pooled, freeze dried, and added to separate culture flasks. Dextrans of the masses 40 kDa, 70 kDa, and 350 kDa were used for external calibration.

5.4.6 Sesquiterpene synthase activity

The sesquiterpene synthase activity was analyzed according to Hartwig et al. (2015). Briefly, 0.2 g washed fungal mycelium was disrupted with 500 µL 50 mM sodium phosphate buffer pH 6.0 using a Precellys 24 bead mill (PEQLAB, Erlangen, Germany). The lysate was incubated with 6.5 µmol farnesyl pyrophosphate ammonium salt (FPP) in a biphasic system with the lysate and 500 µL n-octane. After 8 h at 24 °C, the synthesis of the major product α-ylangene was quantified by means of gas-chromatography-flame ionization detection (GC-FID) with methyl undecanoate (10 mg L^{-1}) as an internal standard. Blanks were carried out by incubating the lysate without substrate and vice versa. The sesquiterpene synthase activity was calculated from the α-ylangene concentration with respect to the wet biomass (U g^{-1} BWM). One Unit was defined as the synthesis of 1 µmol α-ylangene per minute.

5.5 Results

5.5.1 Sesquiterpene synthesis of *T. floriformis*

In preliminary experiments, *T. floriformis* was identified as potent sesquiterpene

producer with a rich product spectrum. This fungus provided a rich volatilome including various sesquiterpenes and sesquiterpenoids (Table 5.5.1). After inoculation, a 2 d lag phase was observed. Afterward, a nonlinear decrease of glucose and dissolved oxygen occurred. Glucose was fully metabolized after 7 d and formation of volatiles started. The maximum *α*-ylangene concentration was detected after 17 d of cultivation followed by a slight decrease.

Identification was based on retention indices, EI-mass spectra*,* and, if available, on external standards. Extracted ion chromatograms (EIC) of m/z = 204, 218, and 220 facilitated the detection of the produced sesquiterpenes as well as alcohols, ketones, and aldehydes derived from them (Figure 5.5.1). Sixteen sesquiterpene hydrocarbons were identified on two stationary phases of different polarity (Table 1). All identified compounds are known fungal metabolites. Besides hydrocarbons, 15 further products, mainly alcohols and aldehydes, but no esters were identified. Some oxygenated products corresponded to the respective hydrocarbons. *α*-Ylangene was the major volatile in the supernatant of *T. floriformis*. Therefore, it was used as a reference compound to evaluate the impact of additives on sesquiterpene synthesis. Further sesquiterpenoids in the culture supernatant showed an analogous variation of concentrations with respect to the additives. As cultivations were carried out in different volumes of liquid culture medium, the influence of the volume on terpene synthesis was examined. The effect of the change of the culture volume from 250 down to 25 mL on product yields was in the same range as the biological batch-to-batch variation (n = 3, ± ≥20%; data not shown).

Table 5.5.1. Volatile sesquiterpenes produced by *T. floriformis* after 21 d.

Peak	Sesquiterpenes	DB-Wax		HP-5 MS		Identification
		RI[a] [-]	R_{Ilit}[b] [-]	RI[a] [-]	R_{Ilit}[b] [-]	
1	*α*-Ylangene	1485	1470	1373	1373	A, B, C, D, E
2	Sativen	1525	1521	1395	1396	A, B, C
3	*α*-Cubebene	1540	1480	1386	1381	A, B, C
4	*β*-Ylangene	1564	1568	1416	1425	A, B, C
5	*β*-Elemene	1571	1573	1379	1392	A, B, C
6	*α*-Bergamotene	1587	1566	1436	1436	A, B, C
7	Germacrene D	1670	1674	1423	1444	A, B, C
8	*β*-Farnesene	1675	1668	1452	1452	A, B, C
9	Bicyclogermacrene	1688	1698	1508	1500	A, B, C
10	*γ*-Cadinene	1700	1725	1481	1477	A, B, C
11	*β*-Cadinene	1713	1720	1518	1518	A, B, C
12	Ledene	1727	1736	1492	1493	A, B, C
13	Germacrene A	1743	1747	1513	1512	A, B, C
14	*α*-Cardinene	1753	1740	1522	1535	A, B, C
15	*β*-Bisabolene	1768	1694	1421	1425	A, B, C
16	Germacrene B	1792	1806	1558	1556	A, B, C
17	(+)-Ledol	2049	2035	1603	1605	A, B, C
18	1-epi-Cadinol	2074	2036	1608	1603	B, C
19	Ledene oxide	2080	2062	1581	-	A, B
20	Cubenol	2062	2084	1637	1643	A, B
21	Ylangenal	2093	-	1676	1674	A, C
22	Ylangenol	2115	-	1666	1666	A, C
23	Sesquiterpene alcohol	2121	-	1680	-	C
24	ent-Germacra-4(15),5,10(14)-trien-1-*β*-ol	2128	-	1687	1686	A, C
25	*α*-Acorenol	2136	2163	1644	1630	A, B, C
26	*τ*-Cadinol	2178	2177	1633	1640	A, B, C
27	*τ*-Muurolol	2185	2187	1651	1648	A, B, C
28	7-epi-*α*-Eudesmol	2206	2205	1669	1651	B, C
29	*α*-Cadinol	2213	2217	1677	1660	A, B, C
30	Sesquiterpene aldehyde	2265	-	1958	-	C
31	Sesquiterpene aldehyde	2294	-	2053	-	C

[a] Retention index calculated according to (van den Dool und Kratz 1963)
[b] Retention index from reference library (National Institute of Standards and Technology)
A: retention index on a polar phase, B: retention index on a non-polar phase, C: comparison of EI mass spectra with the NIST 14 data base, D: reference substance

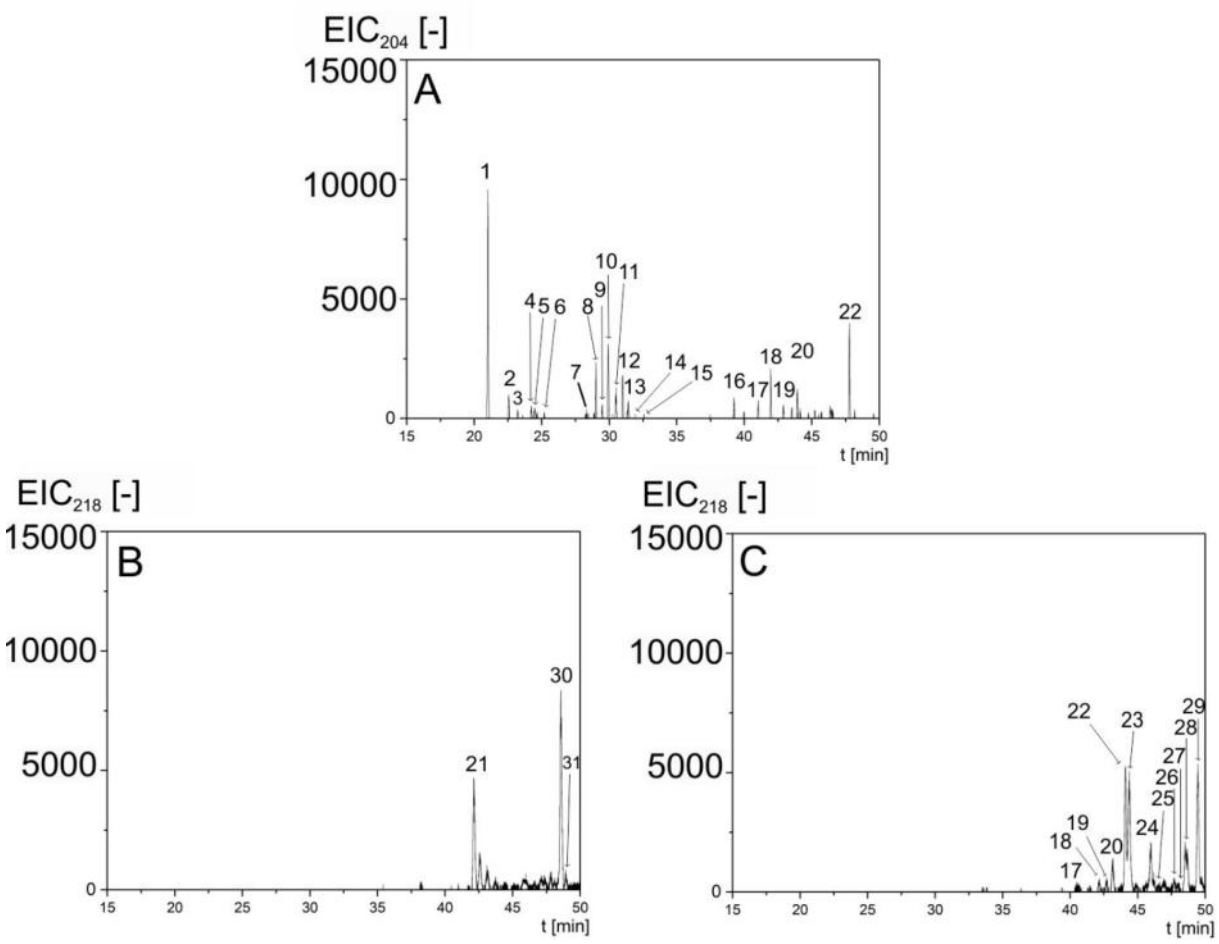

Figure 5.5.1. Extracted ion chromatogram (axes true to scale) of the volatilome of *T. floriformis* (SNL medium, 250 ml, nonbaffled shake flask, 24 °C, 150 rpm, 21 d). Samples were taken *via* sequential Twister extraction and measured using TDS-GC-MS. A: sesquiterpene hydrocarbons (m/z 204), B: sesquiterpene aldehydes (m/z 218) C: sesquiterpene alcohols (m/z 220). Peak numbers refer to table 5.1.1.

Based on their presumed formation mechanism, the identified sesquiterpenes were grouped into four clusters (Figure 5.5.2). The first group contained sesquiterpenes derived from a 1,10-ring closure of the respective *trans*-farnesyl cation, such as germacrene A and *α*-ylangene. Five further products including *α*-, *β*-, and *γ*-cadinene were generated by the same cyclization reaction of the cis farnesyl cation (group II). *β*-Bisabolene and *α*-bergamotene formed the smaller group III derived by 1,6-cyclization of the cis-farnesyl cation. No products derived by 1,6-cyclization of the *trans*-isomer

were identified. The linear *β*-farnesene was formed after loss of a proton. It was classified as product group IV.

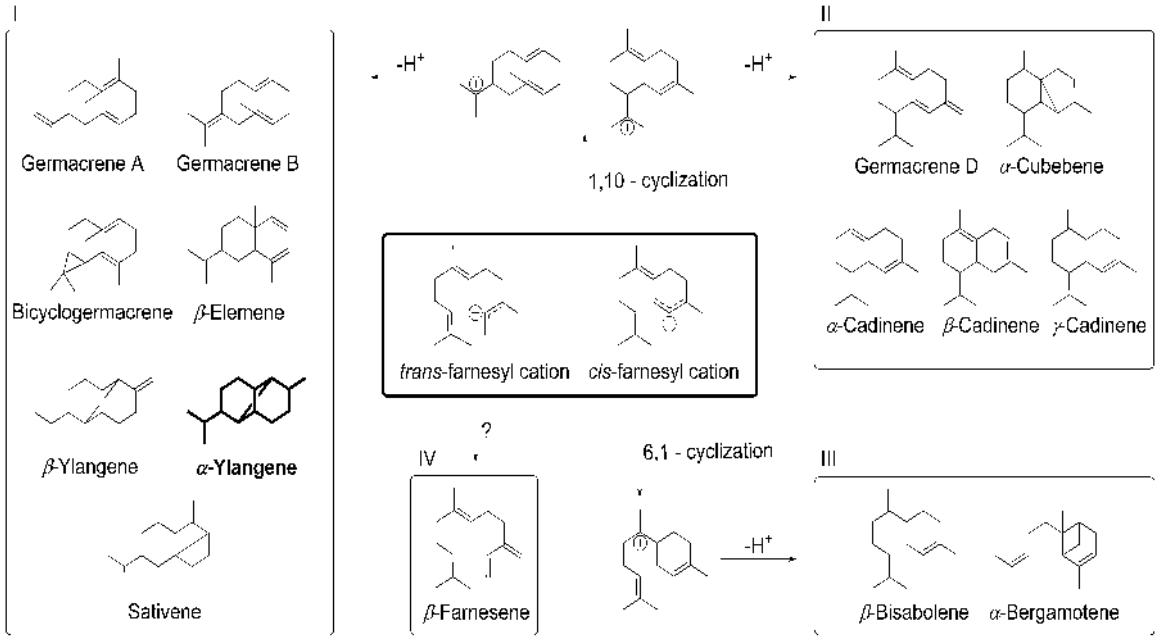

Figure 5.5.2. Clustering of sesquiterpenes of *T. florifomis* based on known cyclization mechanisms (Kramer und Abraham 2012; Schmidt-Dannert 2015; Yoshikuni *et al.* 2006). For groups based on the cyclization mechanism are distinguished. Group I: Sesquiterpenes derived by 1,10-cyclization of the *trans*-farnesyl cation; Group II: Sesquiterpenes derived by 1,10-cyclization of the *cis*-farnesyl cation; Group III: Sesquiterpenes derived by 1,6- cyclization of the *cis*-farnesyl cation; Group IV: Linear farnesene derived by proton loss of the farnesyl cation; Product identification was done after 21 d of submerged cultivation (SNL medium, 250 ml, nonbaffled shake flask, 24 °C, 150 rpm). Products were extracted with sequential SBSE.

5.5.2 Affecting the sesquiterpene synthesis of *T. floriformis*

Submerged cultivations of *T. floriformis* in SNL with various additives did not result in novel products, and qualitatively the volatile profile remained unchanged. However, the overall synthesis rate was influenced. Polysaccharide addition strongly reduced the synthesis of sesquiterpenes and terpenoids (Figure 5.5.3 A). As an example, the effect of rye arabinoxylan was characterized. The feeding of the building blocks of arabinoxylan, namely arabinose, xylose, and ferulic acid had no effect on the sesquiterpene synthesis (3.5 mg L^{-1} α-ylangene), whereas the trisaccharide A^3X

decreased the synthesis significantly by a factor of seven (Figure 5.5.3 B). Addition of arabinoxylan fractions resulted in a decreasing effect, correlated to the polysaccharide chain length: The larger the molecule, the stronger the decrease in α-ylangene concentration.

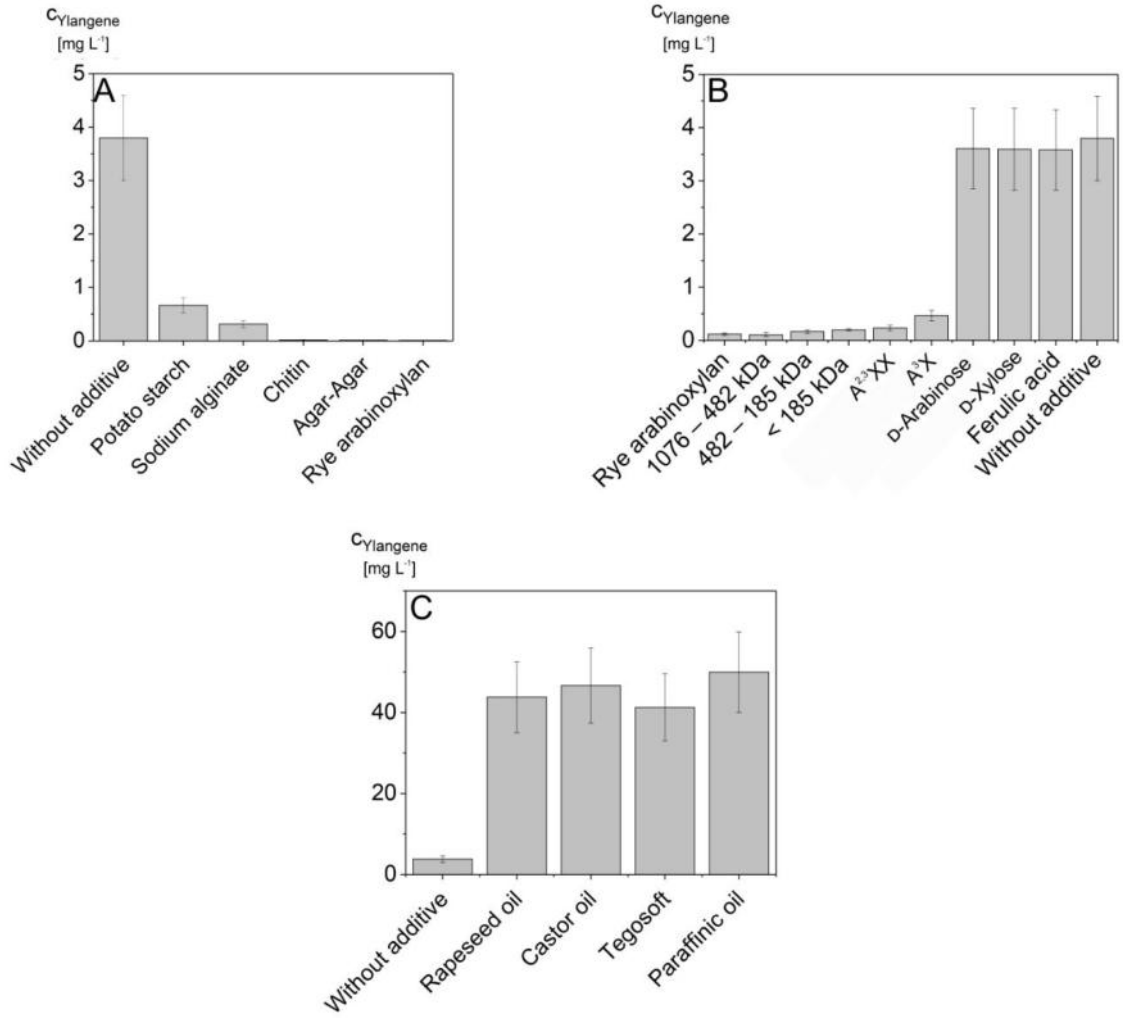

Figure 5.5.3. Impact of effectors on the sesquiterpene synthesis of *T. florifomi*s. A: Polysaccharides (cultivation conditions: SNL medium, 250 ml, non-baffled shaking flask, 24 °C, 150 rpm, 21 days), B: Effect of polysaccharide chain length on the *α*-ylangene yield (cultivation conditions: SNL medium, 50 ml, non-baffled shaking flask, 24 °C, 150 rpm, 21 days), C: Acetyl donors (SNL medium, 250 ml, non-baffled shaking flask, 24 °C, 150 rpm, 21 days).

The addition of lipids resulted in a roughly 10-fold product increase, with *α*-ylangene concentration reaching 46.6 mg L^{-1} after addition of 0.67 % (*v/v*) Tegosoft (Figure 5.5.3

C). Triacylglycerols of different fatty acid composition resulted in similar yields. Feeding free fatty acids had a toxic effect on *T. floriformis* so that neither growth nor product synthesis was detected. A linear correlation with *α*-ylangene synthesis existed for Tegosoft concentrations below 3.5 % (*v/v*). At concentrations above 3.5 %, saturation was reached (Figure 5.5.4). The course of the concentration of dissolved oxygen proved that the addition of a lipophilic phase and subsequent formation of a biphasic system did not adversely affect the oxygen supply of the mycelium growing in the aqueous phase (data not shown).

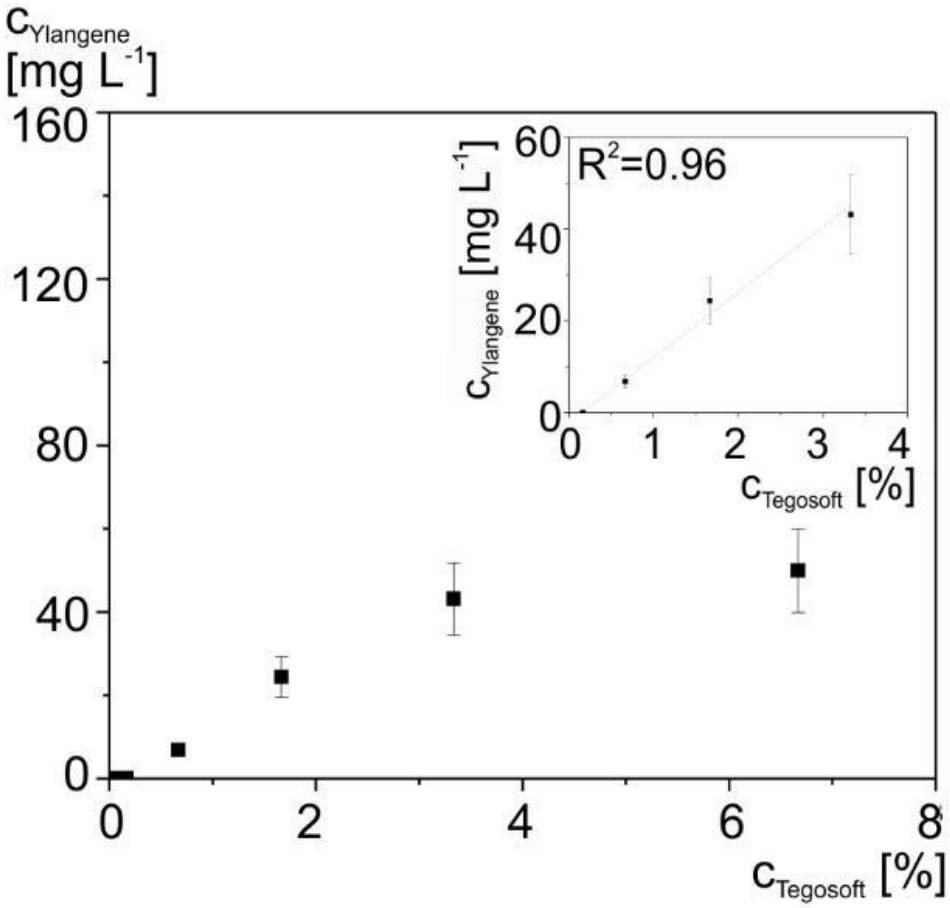

Figure 5.5.4. Correlation between Tegosoft addition and product concentration. *α*-Ylangene was quantified *via* solvent extraction (SNL medium, 50 ml, nonbaffled shake flask, 24 °C, 150 rpm, 21 d).

The activity of the sesquiterpene synthase correlated with the yield of *α*-ylangene (Table 5.5.2). Addition of 3.5 % (*v/v*) Tegosoft led to 53.3 mg L^{-1} *α*-ylangene, the highest concentration reached in this study. Intracellular activity measurement resulted in a maximum substrate turnover of 23.4 µU g^{-1} BWM.

Table 5.5.2. Correlation of intracellular sesquiterpene synthase activity of *T. floriformis* with *α*-ylangene yields after 21 d.

Effect on product yield	Additive	$c_{Ylangene}$ [mg L^{-1}]	$A_{Cyclase}$ [µU g^{-1} BWM]
Decreasing	Rye arabinoxylan	0.01	6.23
-	Without additive	3.80	14.48
Increasing	Tegosoft	53.36	23.43

5.5.3 Validation of effects using other Basidiomycetes

To understand the effects with respect to other organisms, three further fungi were tested. *Postia placenta* and *C. cinerea* are well studied sesquiterpene-producing fungi whereas *C. unicolor* was known as a potent sesquiterpene producer from preliminary experiments (data not shown). *Postia placenta* has 16 sesquiterpene synthase genes (Ichinose und Kitaoka 2018). This fungus was selected based on its genetic identity of 75 % to *T. floriformis* based on ITS sequence data (data not shown). *Coprinopsis cinerea* was selected due to its well characterized sesquiterpene cyclases (Agger *et al.* 2009). *Cerrena unicolor* not only provided a pleasant odor impression during submerged cultivation, it also represented fungi that have a symbiotic lifestyle with insects. This may affect the sesquiterpene biosynthesis differently compared to fungi solely interacting with plants.

Cultivation of these basidiomycete fungi showed specific differences in their response towards the effectors (Table 5.5.3). Tegosoft typically increased the sesquiterpene synthesis except for *Postia placenta*, for which growth was inhibited. Polysaccharide addition resulted in a decreased sesquiterpene synthesis in *T. floriformis*, *P. placenta*, and *C. cinerea*, whereas yields were increased in *C. unicolor* cultures. In the volatilome of *C. unicolor*, the increase of products differed with the additive. Whereas Tegosoft exclusively boosted the synthesis of the 1,10 ring-closure product *α*-muurolen, rye arabinoxylan increased the yield of several sesquiterpenoids.

Table 5.5.3. Impact of effectors on the sesquiterpene synthesis on cultivation day 21 of four basidiomycete species.

Additive	***Tyromyces floriformis***	***Cerrena unicolor***	***Postia placenta***	***Coprinopsis cinerea***
No additive	Reference Synthesis (Blank)		No Synthesis	
Rye arabinoxylan	Synthesis decreased	Synthesis increased	Synthesis decreased slightly	No Synthesis
Tegosoft	Synthesis increased	Synthesis increased	No Synthesis[A]	Synthesis increased

[A] Growth inhibited by the additive

5.6 Discussion

5.6.1 Synthesis of volatiles by *T. floriformis*

Submerged cultivation of *T. floriformis* led to the synthesis of numerous sesquiterpenes after cultivation day 17 (Table 5.5.1). Dissolved oxygen data indicated that the synthesis of sesquiterpenes started after glucose was depleted and the fungal growth was terminated (Figure 5.4.1). It has been reported that the enzymes involved into the mevalonate pathway became active in senescent cells during the late growth phase

only (Kramer und Abraham 2012).

Products derived by 1,10 cyclization of the farnesyl cation predominated the product spectrum (Figure 5.5.2). The majority of products were classified as group I and the second most abundant as group II. These two groups only differ regarding the isomer of the cyclization educt. The *trans*-farnesyl cation is rearranged to the *cis*-form before further reaction to products of group II. Sesquiterpene synthases are known to perform different cyclizations and isomerizations. The enzyme can thus create a highly diverse product spectrum (Yoshikuni *et al.* 2006). β-Farnesene (group IV), β-bisabolene (group III), germacrene D (group II) as well as sativene, α- and β-ylangene (group I) were described as reaction products of a plant γ-humulene synthase, whereas β-bisabolene (group III), germacrene D and α-cadinene (group II) as well as germacrene A, B, and α-ylangene (group I) and are known products of a δ-selinene synthase (Steele *et al.* 1998). Due to the diverse product spectrum present in *T. floriformis*, at least two sesquiterpene synthases are expected in this fungus.

5.6.2 Effectors of the sesquiterpene synthesis

Terpenes are produced to deal with diverse ecological challenges. Therefore, their synthesis is regulated with respect to the requirements of the surroundings (Rohlfs und Churchill 2011). They are known to be highly regulated on genetic and cellular levels, even though effectors are poorly understood still (Hoffmeister und Keller 2007; Chanda *et al.* 2009). All polysaccharides, regardless of their different chemical building blocks, inhibited the sesquiterpene synthesis of three out of the four basidiomycetes investigated (Table 3). These effectors occur in plant storage compartments, in the plant and algal cell wall, or constitute the exoskeleton of insects. Basidiomycete cells are supposed to get in regular contact with these ubiquitous compounds.

Lignocellulose and hemicelluloses provide the regular growth matrix of xylophilic fungi. It may be speculated that during the coevolution of wood degrading fungi and their plant feedstocks, the fungi have attempted to reinforce the attack by the secreted enzymes by producing hydrophobic volatiles toxic for the plant. The inhibitory effect of polysaccharides, thus, would add to the merely mechanical protection they provide (Figure 5.5.3 A).

The molecular mass of additives correlated with the inhibitory effect (Figure 5.5.3 B). Whereas monosaccharides or ferulic acid, both potential immediate carbon sources, did not inhibit the sesquiterpene synthesis, dimers *e.g.* A^3X were already large enough to inhibit the *α*-ylangene synthesis by a factor of 7.

This inhibitory effect was not found for *Cerrena unicolor.* The fungus is known for a symbiotic lifestyle with the wasps *Tremex columbia* and *Megarhyssa spec,* which are using terpenes for intraspecies communication (Kuo und Methven 2010). Thus, it may be speculated that fungi preferably interacting with lignified plants were inhibited by polysaccharides, whereas fungi depending on an interaction with insects produce higher concentrations of attracting sesquiterpenes. Hence, the results would agree with the plant–insect–fungus interactions described by Kramer and Abraham (2012) (Table 5.5.3).

The addition of glycerol-bound fatty acids to a fungal culture may cause multiple effects, including direct precursor activity through acetate supply, or indirect effector activity, or simply serving as a lipophilic accumulation site for the lipophilic terpene products (Berg et al., 2014) (Figure 5.5.3 C). The linear correlation of low to medium Tegosoft concentrations and *α*-ylangene concentration in *T. floriformis* points to a more

and more filled-up acetate pool (Figure 5.5.4). The sesquiterpene synthase activity was increased compared to the cultivation without acetyl donors (Table 5.5.2).

When lipids were added in higher concentration, fine droplets remaining at the end of the cultivation were visually detectable. In these cultures, the cytotoxic effects of higher concentrations of volatile terpenes on the producer cells will be decreased by shifting the concentration equilibria to the lipophilic phase. An overlapping precursor or effector role may have added to the observed increase of the sesquiterpene concentration. In the case of paraffinic oil, the provision of a lipophilic accumulation site is the most likely explanation. It is difficult to design experiments allowing the quantitative differentiation of the three different mechanisms. Labelled acetate is too central and recovered from most metabolite fractions upon some hours of incubation. However, the cyclase activating effect of Tegosoft cannot be denied (Table 5.5.2), and this was shown for three out of the four basidiomycetes (Table 5.5.3). The exception to the rule was *Postia placenta*, which did not grow in the biphasic medium, most likely because of a secreted lipase resulting in cytotoxic intermediate levels of fatty acids thus liberated.

5.7 Conclusion

Various polysaccharides and acetyl donors, when added to the cultivation medium of four basidiomycete species, affected the sesquiterpene synthesis. Polysaccharides decreased the sesquiterpene yield for phytopathogenic fungi, whereas acetyl donors boosted the yields by a factor of 10 or more. This should be considered, if complex side streams from agroindustrial sources, such as fruit peel, husks, oil press cake or the like are used to replace the expensive pure components of the nutrient media. The results correlated with the measured sesquiterpene cyclase activity. Even though some effectors affecting the activity of this key enzyme were identified, the causal

relationships are not yet fully understood. Experiments to study transcriptomics as well as the characterization of the isolated cyclase will follow.

6 Abschluss und Ausblick

Die vorliegende Arbeit bietet neue Erkenntnisse über die *de-novo*-Biosynthese von acetogenen Aromastoffen in Basidiomyceten. Dabei wurde im ersten Teil der Arbeit mit (5*E*/*Z*,7*E*,9)-Decatrien-2-on ein neuer Naturstoff im Kulturüberstand des Birkenporlings *Fomitopsis betulina* identifiziert. Mit seinem klaren ananasartigen Geruchseindruck stellt diese Verbindung ein besonders beliebtes Fruchtaroma dar. Besonders das (5*Z*)-Isomer besitzt eine niedrige Geruchsschwelle von 0,028 ng (GC-O). Supplementierung des Kulturmediums mit 1/2-^{13}C markierten Acetat, bewies eindeutig den Aufbau über die Polyketid-Biosynthese.

(5*E*/*Z*,7*E*,9)-Decatrien-2-on ist ein ungewöhnliches Polyketid. Das geradzahlige Methylketon besitzt an der fünften, siebten und neunten Stelle, jedoch nicht an der dritten eine Doppelbindung. Weiterhin ist auffällig, dass, anders als üblich, weder ein Hetero- noch ein Makrocyclus gebildet wird. Lineare Polyene werden üblicherweise als freie Säure von den PKS freigesetzt.

Der Mechanismus der Polyketid-Biosynthese zur Produktion von (5*E*/*Z*,7*E*,9)-Decatrien-2-ons sollte in weiterführenden Arbeiten geklärt werden. Von besonderem Interesse ist die Identifizierung der Startereinheit. Vermutet wird die Bildung eines geradzahligen Polyens über eine ungeradzahlige Startereinheit. Dabei entsteht durch Kettenverlängerung eine ungeradzahlige Säure. Decarboxyliert diese, entsteht ein geradzahliges Methylketon. Anhand dieses Mechanismus wäre jedoch weder die endständige Doppelbindung noch der Grad der Reduktion des dritten Kohlenstoffs zu erklären. Eine Alternative wäre eine Abspaltung der Säuregruppe mit Hilfe einer Decarboxylase.

Weiterhin sollte der PKS Multienzymkomplex identifiziert werden. Bisher ist unklar, ob (5*E*/*Z*,7*E*,9)-Decatrien-2-on das finale Produkt ist oder, wie in Kapitel 1.2.5

beschrieben, ein Polyketid ist, welches vorzeitig aus dem Multienzymkomplex entlassen wird. Dabei wäre es möglich, dass das (5*E*/*Z*,7*E*,9)-Decatrien-2-on ein Intermediat eines größeren Polyens wie Piptoporinsäure oder Laetiporinsäure ist.

Im zweiten Teil der Arbeit wurde der Einfluss der Medienzusammensetzung auf die Sesquiterpensynthaseproduktion gezeigt. Positive Effekte durch die Zugabe von Ölen sowie Sesquiterpenkonzentration reduzierende Effekte von Polysacchariden korrelierten direkt mit der Synthase-Aktivität. Bisher sind die zellulären Mechanismen noch nicht geklärt.

Medienadditive könnten sowohl die Enzymproduktion beeinflussen als auch die Enzymaktivität direkt regulieren. Um diese Effekte weiter beschreiben zu können, müssen in weiterführenden Arbeiten die Sesquiterpencyclasen identifiziert und isoliert werden. Anschließend kann der Einfluss der Additive näher charakterisiert werden. Einflüsse auf die Genexpression könnten über quantitative Polymerase-Kettenreaktion analysiert werden. Biotransformastionsreaktionen mit dem gereinigten Enzyms in Anwesenheit der Effektoren würden den direkten Einfluss auf die Enzymaktivität beleuchten.

7 Literaturverzeichnis

Abraham, B. G.; Berger, R. G. (1994): Higher fungi for generating aroma components through novel biotechnologies. In: *J. Agric. Food Chem.* 42 (10), S. 2344–2348. DOI: 10.1021/jf00046a050.

Abraham, W. R. (2001): Bioactive sesquiterpenes produced by fungi: are they useful for humans as well? In: *Curr. Med. Chem.* 8 (6), S. 583–606. DOI: 10.2174/0929867013373147.

Adda, J.; Gripon, J. C.; Vassal, L. (1982): The chemistry of flavour and texture generation in cheese. In: *Food Chem.* 9 (1-2), S. 115–129. DOI: 10.1016/0308-8146(82)90073-5.

Agger, S.; Lopez-Gallego, F.; Schmidt-Dannert, C. (2009): Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. In: *Mol. Microbiol.* 72 (5), S. 1181–1195. DOI: 10.1111/j.1365-2958.2009.06717.x.

Anderson, B.; Terblanche, J. S.; Ellis, A. G. (2010): Predictable patterns of trait mismatches between interacting plants and insects. In: *BMC Evol. Biol.* 10, Nr. 204. DOI: 10.1186/1471-2148-10-204.

Ara, V.; Heil, M. (2006): Authentizität von Ananasaroma – Eine Betrachtung der Ester, In: Gesellschaft für Lebensmittelforschung mbH (Hrsg.), *Flüssiges Obst*, 73 (5) S. 245–249.

Augustyn, O.P.H.; Marais, J. (1982): Aroma Components in Freon Extracts of Wine. In: *SAJEV* 3 (1), S. 37–39. DOI: 10.21548/3-1-2393.

Baer, P.; Rabe, P.; Citron, C. A.; de Oliveira Mann, C. C.; Kaufmann, N.; Groll, M.; Dickschat, J. S. (2014): Hedycaryol synthase in complex with nerolidol reveals terpene cyclase mechanism. In: *Chembiochem* 15 (2), S. 213–216. DOI: 10.1002/cbic.201300708.

Barba, C.; Thomas-Danguin, T.; Guichard, E. (2017): Comparison of stir bar sorptive extraction in the liquid and vapour phases, solvent-assisted flavour evaporation and headspace solid-phase microextraction for the (non)-targeted analysis of volatiles in fruit juice. In: *LWT - Food Sci. Technol.* 85, S. 334–344. DOI: 10.1016/j.lwt.2016.09.015.

Beauchne, D.; Grua-Priol, J.; Lamer, T.; Demaimay, M.; Qumeneur, F. (2000): Concentration by pervaporation of aroma compounds from *Fucus serratus*. In: *J. Chem. Technol. Biotechnol.* 75 (6), S. 451–458. DOI: 10.1002/1097-4660(200006)75:6<451::AID-JCTB231>3.0.CO;2-U.

Beck, J.; Ripka, S.; Siegner, A.; Schiltz, E.; Schweizer, E. (1990): The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. In: *Eur. J. Biochem.* 192 (2), S. 487–498. DOI: 10.1111/j.1432-1033.1990.tb19252.x.

Belitz, H. D.; Grosch, W.; Schieberle, P. (2008): Lehrbuch der Lebensmittelchemie. 6th Ed. Berlin, Heidelberg: Springer (Springer-Lehrbuch). Online verfügbar unter http://dx.doi.org/10.1007/978-3-540-73202-0.

Bel-Rhlid, R.; Berger, R. G.; Blank, I. (2018): Bio-mediated generation of food flavors – Towards sustainable flavor production inspired by nature. In: *Trends Food Sci. Technol.* 78, S. 134–143. DOI: 10.1016/j.tifs.2018.06.004.

Ben Akacha, N.; Gargouri, M. (2015): Microbial and enzymatic technologies used for the production of natural aroma compounds. Synthesis, recovery modeling, and bioprocesses. In: *Food Bioprod. Process.* 94, S. 675–706. DOI: 10.1016/j.fbp. 2014.09.011.

Berger, R. G.; Kollmannsberger, H. (1985): Pineapple Flavours - Biosynthetic routes and sensorial properties. In: R. G. Berger, S. Nitz und P. Schreier (Hrsg.), *Proceeding of Topics in Flavor Research*, S. 305–320.

Berger, R. G.; Drawert, F.; Kollmannsberger, H.; Nitz, S.; Schraufstetter, B. (1985): Novel volatiles in pineapple fruit and their sensory properties. In: *J. Agric. Food Chem.* 33 (2), S. 232–235. DOI: 10.1021/jf00062a019.

Berger, R. G.; Zorn, H. (2004): Flavors and Fragrances. In: Tkacz, J. S. und Lange, L. (Hrsg.): Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine, Bd. 63. Boston, MA: Springer US; Imprint; Springer, S. 341–358.

Birch, A. J.; Donovan, F. W. (1953): Studies in relation to Biosynthesis. I. Some possible routes to derivatives of Orcinol and Phloroglucinol. In: *Aust. J. Chem.* 6 (4), S. 360. DOI: 10.1071/CH9530360.

Bisang, C.; Long, P. F.; Cortés, J.; Westcott, J.; Crosby, J.; Matharu, A. L. (1999): A chain initiation factor common to both modular and aromatic polyketide synthases. In: *Nature* 401 (6752), S. 502–505. DOI: 10.1038/46829.

Bohlmann, F. (1984): Encyclopaedia of the terpenoids. Band 1: A–H, Band 2: I–Z. In: *Angew. Chem.* 96 (4), S. 313–314. DOI: 10.1002/ange.19840960429.

Böker, A.; Fischer, M.; Berger, R. G. (2001): Raspberry ketone from submerged cultured cells of the basidiomycete *Nidula niveo-tomentosa*. In: *Biotechnol. Prog.* 17 (3), S. 568–572. DOI: 10.1021/bp010011b.

Boland, W.; Mertes, K. (1985): Biosynthesis of algal pheromones. A model study with the composite *Senecio isatideus*. In: *Eur. J. Biochem.* 147 (1), S. 83–91. DOI: 10.1111/j.1432-1033.1985.tb08722.x.

Borup, B.; Daussmann, T.; Collier, S. (2010): Chiral Alcohols by Enzymatic Preparation. In: Howard Loxton und Keith Lye (Hrsg.): Encyclopedia of Industrial Biotechnology. J. Wiley & Sons Canada, S. 1377. DOI: 10.1002/9780470054581.eib215.

Brenna, E.; Parmeggiani, F. (2017): Biotechnological Production of Flavors. In: Christoph Wittmann und James C. Liao (Hrsg.): Industrial Biotechnology, Bd. 1. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KG, S. 271–308.

Brown, D. W.; Butchko, R. A. E.; Busman, M.; Proctor, R. H. (2012): Identification of gene clusters associated with fusaric acid, fusarin, and perithecial pigment production in *Fusarium verticillioides*. In: *Fungal Genet. Boil.: FG & B* 49 (7), S. 521–532. DOI: 10.1016/j.fgb.2012.05.010.

Bruyne, M. de; Baker, T. C. (2008): Odor detection in insects: volatile codes. In: *J. Chem. Ecol.* 34 (7), S. 882–897. DOI: 10.1007/s10886-008-9485-4.

Buchanan, B. B.; Gruissem, W.; Jones, R. L. (Hrsg.) (2015): Biochemistry & molecular biology of plants. 2nd ed. Hoboken, NJ, USA: John Wiley & Sons, Inc Online verfügbar unter http://gbv.eblib.com/patron/FullRecord.aspx?p=2083724.

Cane, D. E.; Shim, J. H.; Xue, Q.; Fitzsimons, B. C.; Hohn, T. M. (1995): Trichodiene synthase. Identification of active site residues by site-directed mutagenesis. In: *Biochemistry* 34 (8), S. 2480–2488.

Chanda, A.; Roze, L. V.; Kang, S.; Artymovich, K. A.; Hicks, G. R.; Raikhel, N. V. (2009): A key role for vesicles in fungal secondary metabolism. In: *Proc. Natl. Acad. Sci.* 106 (46), S. 19533–19538. DOI: 10.1073/pnas.0907416106.

Chen, X.; Zhang, Y.; Zu, Y.; Yang, L. (2012): Chemical composition and antioxidant activity of the essential oil of *Schisandra chinensis* fruits. In: *Nat. Prod. Res.* 26 (9), S. 842–849. DOI: 10.1080/14786419.2011.558016.

Christen, P.; Meza, J. C.; Revah, S. (1997): Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*. Influence of the substrate type and the presence of precursors. In: *Mycol. Res.* 101 (8), S. 911–919. DOI: 10.1017/S0953756297003535.

Činčala, L.; Illeová, V.; Antošová, M.; Štefuca, V.; Polakovič, M. (2015): Investigation of plant sources of hydroperoxide lyase for 2(*E*)-hexenal production. In: *Acta Chim. Slov.* 8 (2), S. 156–165. DOI: 10.1515/acs-2015-0027.

Civjan, N. (Hrsg.) (2012): Natural Products in Chemical Biology. 1st ed. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Coconuts Manila (2015): Chanel needs more ylang-ylang for N°5 perfume. zuletzt geprüft am 10.05.2018.

Collie, N.; Myers, W. S. (1893): VII.—The formation of orcinol and other condensation products from dehydracetic acid. In: *J. Chem. Soc., Trans.* 63 (0), S. 122–128. DOI: 10.1039/CT8936300122.

Cox, R. J. (2007): Polyketides, proteins and genes in fungi. Programmed nano-machines begin to reveal their secrets. In: *Org. Biomol. Chem.* 5 (13), S. 2010–2026. DOI: 10.1039/B704420H.

Cox, R. J.; Simpson, T. J. (2009): Chapter 3 Fungal Type I Polyketide Synthases. In: Russell J. Cox and Thomas J. Simpson (Hrsg.): Complex Enzymes in Microbial Natural Product Biosynthesis, Part B: Polyketides, Aminocoumarins and Carbohydrates, Bd. 459: Elsevier (Methods in Enzymology), S. 49–78.

Crawford, J. M.; Thomas, P. M.; Scheerer, J. R.; Vagstad, A. L.; Kelleher, Neil L.; Townsend, C. A. (2008): Deconstruction of iterative multidomain polyketide synthase function. In: *Science (New York, N.Y.)* 320 (5873), S. 243–246. DOI: 10.1126/science.1154711.

Davis, E. M.; Croteau, R. (2000): Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes, and Diterpenes. In: Leeper, F. J. und Vederas J. C. (Hrsg.): Biosynthesis. Aromatic Polyketides, Isoprenoids, Alkaloids, Bd. 209. Berlin, Heidelberg: Springer (Topics in Current Chemistry, 209), S. 53–95.

Dickschat, J. S. (2017): Fungal volatiles - a survey from edible mushrooms to moulds. In: *Nat. Prod. Rep.* 34 (3), S. 310–328. DOI: 10.1039/c7np00003k.

Dravnieks, A. (1985): Atlas of odor character profiles. Philadelphia, Pa.: ASTM data series publication, Bd 61.

Falconnier, B.; Lapierre, C.; Lesage-Meessen, L.; Yonnet, G.; Brunerie, P.; Colonna-Ceccaldi, B. (1994): Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937. Identification of metabolic pathways. In: *J. Biotechnol.* 37 (2), S. 123–132. DOI: 10.1016/0168-1656(94) 90003-5.

Ferrer, J. L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. (1999): Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. In: *Nat. Struct. Boil.* 6 (8), S. 775–784. DOI: 10.1038/11553.

Fraatz, M. A.; Riemer, S. J. L.; Stöber, R.; Kaspera, R.; Nimtz, M.; Berger, R. G.; Zorn, H. (2009): A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone. In: *J. Mol. Cat. B-Enzym.* 61 (3-4), S. 202–207. DOI: 10.1016/j.molcatb.2009.07.001.

Fraga, B. M. (2013): Natural sesquiterpenoids. In: *Nat. Prod. Rep.* 30 (9), S. 1226–1264. DOI: 10.1039/C3NP70047J.

Freihorst, D.; Brunsch, M.; Wirth, S.; Krause, K.; Kniemeyer, O.; Linde, J. (2018): Smelling the difference. Transcriptome, proteome and volatilome changes after mating. In: *Fungal Genet. Biol.* 112, S. 2–11. DOI: 10.1016/j.fgb.2016.08.007.

George, K. W.; Alonso-Gutierrez, J.; Keasling, J. D.; Lee, T. S. (2015): Erratum to: Isoprenoid Drugs, Biofuels, and Chemicals--Artemisinin, Farnesene, and Beyond. In: *Adv. Biochem. Eng./ Biotechnol.* 148, S. 469. DOI: 10.1007/10_2015_310.

Gershenzon, J.; Dudareva, N. (2007): The function of terpene natural products in the natural world. In: *Nat. Chem. Biol.* 3 (7), S. 408–414. DOI: 10.1038/nchembio. 2007.5.

Gill, M. (1982): Polyolefinic 18-methyl-19-oxoicosenoic acid pigments from the fungus *Piptoporus australiensis* (Wakefield) Cunningham. In: *J. Chem. Soc., Perkin Trans. 1*, S. 1449–453. DOI: 10.1039/P19820001449.

Grishin, A. A.; Lutskii, V. I.; Penzina, T. A.; Dudareva, L. V.; Zorina, N. V.; Polyakova, M. S.; Osipenko, S. N. (2016): Composition of the Supercritical CO_2 Extract of the Fungus *Piptoporus betulinus*. In: *Chem. Nat. Compd.* 52 (3), S. 436–440. DOI: 10.1007/s10600-016-1667-7.

Grosse, M.; Pendzialek, T.; Berger, R. G.; Krings, U. (2019): (5*E*/*Z*,7*E*,9)-Decatrien-2-ones, Pineapple-like Flavors from *Fomitopsis betulina* - Structure Elucidation and Sensorial Properties. In: *J. Agric. Food Chem.* DOI: 10.1021/acs.jafc.9b06105.

Habermehl, G.; Hammann, P. E.; Krebs, H. C. (2002): Naturstoffchemie. Eine Einführung. Berlin, Heidelberg: Springer Berlin Heidelberg.

Halls, S. C.; Gang, D. R.; Weber, D. J. (1994): Seasonal variation in volatile secondary compounds of *Chrysothamnus nauseosus* (Pallas) britt.; asteraceae ssp.hololeucus (Gray) hall. & clem. Influences herbivory. In: *J. Chem. Ecol.* 20 (8), S. 2055–2063. DOI: 10.1007/BF02066242.

Hartwig, S.; Frister, T.; Alemdar, S.; Li, Z.; Scheper, T.; Beutel, S. (2015): SUMO-fusion, purification, and characterization of a (+)-zizaene synthase from *Chrysopogon zizanioides*. In: *Biochem. Biophys. Res. Commun.* 458 (4), S. 883–889. DOI: 10.1016/j.bbrc.2015.02.053.

Hawke, J. C. (1966): Section D. Dairy chemistry. The formation and metabolism of methyl ketones and related compounds. In: *J. Dairy Res.* 33 (2), S. 225–243. DOI: 10.1017/S0022029900011894.

He, H.-Y.; Yuan, H.; Tang, M.-C.; Tang, G.-L. (2014): An unusual dehydratase acting on glycerate and a ketoreductase stereoselectively reducing α-ketone in polyketide starter unit biosynthesis. In: *Angew. Chem., Int. Ed.* 53 (42), S. 11315–11319. DOI: 10.1002/anie.201406602.

He, J.; Hertweck, C. (2004): Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin. Involvement of an unprecedented N-oxygenase. In: *J. Am. Chem. Soc.* 126 (12), S. 3694–3695. DOI: 10.1021/ja039328t.

Hertweck, C. (2009): The biosynthetic logic of polyketide diversity. In: *Angew. Chem. Int. Ed. Engl.* 48 (26), S. 4688–4716. DOI: 10.1002/anie.200806121.

Hertweck, C.; Jarvis, A. P.; Xiang, L.; Moore, B. S.; Oldham, N. J. (2001): A Mechanism of Benzoic Acid Biosynthesis in Plants and Bacteria that Mirrors Fatty Acid *β*-Oxidation. In: *ChemBioChem* 2 (10), S. 784. DOI: 10.1002/1439-7633 (20011001)2:10<784::aid-cbic784>3.0.co;2-k.

Herz, W.; Grisebach, H.; Kirby, G. W.; Tamm, Ch.; Gill, M.; Steglich, W. (1987): Fortschritte der Chemie organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products. Vienna: Springer Vienna (51).

Hirschmann, R.; Reule, W.; Oppenländer, T.; Baganz, F.; C. Hass, V. (2019): Integrating Whole Cell Biotransformation of Aroma Compounds into a Novel Biorefinery Concept. In: Beschkov V. (Hrsg.): Biorefinery Concepts, London: IntechOpen. S. 1–22. DOI: 10. 5772/intechopen.88158

Hoffmeister, D.; Keller, N. P. (2007): Natural products of filamentous fungi. Enzymes, genes, and their regulation. In: *Nat. Prod. Rep.* 24 (2), S. 393–416. DOI: 10.1039/b603084j.

Hopwood, D. A. (1997): Genetic Contributions to Understanding Polyketide Synthases. In: *Chem. Rev.* 97 (7), S. 2465–2498. DOI: 10.1021/cr960034i.

Horinouchi, S. (2008): Combinatorial biosynthesis of non-bacterial and unnatural flavonoids, stilbenoids and curcuminoids by microorganisms. In: *J. Antibiot.* 61 (12), S. 709–728. DOI: 10.1038/ja.2008.85.

Hybelbauerová, S.; Sejbal, J.; Dracínský, M.; Hahnová, A.; Koutek, B. (2008): Chemical constituents of *Stereum subtomentosum* and two other birch-associated basidiomycetes. An interspecies comparative study. In: *Chem. Biodiversity* 5 (5), S. 743–750. DOI: 10.1002/cbdv.200890070.

Hynes, J.; Müller, C. T.; Jones, T. H.; Boddy, L. (2007): Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. In: *J. Chem. Ecol.* 33 (1), S. 43–57. DOI: 10.1007/s10886-006-9209-6.

Ichinose, H.; Kitaoka, T. (2018): Insight into metabolic diversity of the brown-rot basidiomycete *Postia placenta* responsible for semi-comprehensive screening of

cytochrome P450 monooxygenase involved in protoilludene metabolism sesquiterpene biosynthesis. In: *Microb. Biotechnol.* 11 (5), S. 952–965. DOI: 10.1111/1751-7915.13304.

Ikeda, H.; Nonomiya, T.; Usami, M.; Ohta, T.; Omura, S. (1999): Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. In: *Proceedings of the National Academy of Sciences of the United States of America* 96 (17), S. 9509–9514. DOI: 10.1073/pnas.96.17.9509.

International Flavors & Fragrances Inc. (2018): International Flavors & Fragrances - 10K Report 2017 03, zuletzt geprüft am 10.09.2018.

Inoue, Y.; Shiraishi, A.; Hada, T.; Hirose, K.; Hamashima, H.; Shimada, J. (2004): The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. In: *FEMS Microbiol. Lett.* 237 (2), S. 325–331. DOI: 10.1111/j.1574-6968.2004.tb09714.x

Jansen, B. J. M.; de Groot, A. (2004): Occurrence, biological activity and synthesis of drimane sesquiterpenoids. In: *Nat. Prod. Rep.* 21 (4), S. 449–477. DOI: 10.1039/b311170a.

Jeleń, H. H.; Majcher, M.; Dziadas, M. (2012): Microextraction techniques in the analysis of food flavor compounds. A review. In: *Anal. Chim. Acta* 738, S. 13–26. DOI: 10.1016/j.aca.2012.06.006.

Jeromin, G.; Bertau, M. (2005): Bioorganikum. Praktikum der Biokatalyse. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KG.

Jez, Joseph M.; Bowman, Marianne E.; Noel, Joseph P. (2002): Expanding the biosynthetic repertoire of plant type III polyketide synthases by altering starter molecule specificity. In: *Proceedings of the National Academy of Sciences of the United States of America* 99 (8), S. 5319–5324. DOI: 10.1073/pnas.082590499.

Jin, J.; Kim, M. J.; Dhandapani, S.; Tjhang, J. G.; Yin, J.-L.; Wong, L. (2015): The floral transcriptome of ylang ylang (*Cananga odorata* var. *fruticosa*) uncovers biosynthetic pathways for volatile organic compounds and a multifunctional and novel sesquiterpene synthase. In: *J. Exp. Bot.* 66 (13), S. 3959–3975. DOI: 10.1093/jxb/erv196.

Jordán, M. J.; Margaría, C. A.; Shaw, P. E.; Goodner, K. L. (2002): Aroma active components in aqueous kiwi fruit essence and kiwi fruit puree by GC-MS and multidimensional GC/GC-O. In: *J. Agric. Food Chem.* 50 (19), S. 5386–5390. DOI: 10.1021/jf020297f.

Jurado, J. M.; Ballesteros, O.; Alcázar, A.; Pablos, F.; Martín, M. J.; Vílchez, J. L.; Navalón, A. (2007): Characterization of aniseed-flavoured spirit drinks by headspace solid-phase microextraction gas chromatography-mass spectrometry and chemometrics. In: *Talanta* 72 (2), S. 506–511. DOI: 10.1016/j.talanta.2006.11.008.

Kinderlerer, J. L. (1993): Fungal strategies for detoxification of medium chain fatty acids. In: *Int. Biodeter. Biodegr.* 32 (1-3), S. 213–224. DOI: 10.1016/0964-8305(93)90053-5.

Klein, A.; Hilmer, J.-M. (2013): Steroide und Aromastoffe. In: Sahm H., Antranikian G., Stahmann K.-P. und Takors R. (Hrsg.): Industrielle Mikrobiologie, Bd. 124. Berlin, Heidelberg: Springer Berlin Heidelberg, S. 245–270.

Kramer, R.; Abraham, W.-R. (2012): Volatile sesquiterpenes from fungi. What are they good for? In: *Phytochem. Rev.* 11 (1), S. 15–37. DOI: 10.1007/s11101-011-9216-2.

Krammer, G. (2020): Biotechnologische Aromastoffproduktion in der Industrie. Hannover, 27.01.2020. E-mail Korrespondenz mit M. Große.

Krings, U.; Berger, R. G. (1998): Biotechnological production of flavours and fragrances. In: *Appl. Microbiol. Biotechnol.* 49 (1), S. 1–8. DOI: 10.1007/s002530051129.

Kunert, G.; Otto, S.; Röse, U. S. R.; Gershenzon, J.; Weisser, W. W. (2005): Alarm pheromone mediates production of winged dispersal morphs in aphids. In: *Ecol. Lett.* 8 (6), S. 596–603. DOI: 10.1111/j.1461-0248.2005.00754.x.

Kuo, M.; Methven, A. S. (2010): 100 cool mushrooms. 1st ed. Michigan: The University of Michigan Press.

Lee, S.-Y.; Kim, M.; Kim, S.-H.; Hong, C.-Y.; Ryu, S.-H.; Choi, I.-G. (2016): Transcriptomic analysis of the white rot fungus *Polyporus brumalis* provides insight

into sesquiterpene biosynthesis. In: *Microbiol. Res.* 182, S. 141–149. DOI: 10.1016/j.micres.2015.10.008.

Leeper, F. J.; Vederas, J. C. (Hrsg.) (2000): Biosynthesis. Aromatic Polyketides, Isoprenoids, Alkaloids. 1st ed. Berlin, Heidelberg: Springer. Online verfügbar unter http://dx.doi.org/10.1007/3-540-48146-X.

Leonhardt, R.-H.; Berger, R. G. (2015): Nootkatone. In: *Adv. Biochem. Engin./Biotechnol.* 148, S. 391–404. DOI: 10.1007/10_2014_279.

Lesburg, C. A. (1997): Crystal structure of pentalenene synthase. Mechanistic Insights on Terpenoid Cyclization Reactions in Biology. In: *Science* 277 (5333), S. 1820–1824. DOI: 10.1126/science.277.5333.1820.

Lin, B.; Tao, Y. (2017): Whole-cell biocatalysts by design. In: *Microb. Cell Fact.* 16 (1), S. 106. DOI: 10.1186/s12934-017-0724-7.

Liu, K.-S. (1994): Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. In: *J. Am. Oil Chem. Soc.* 71 (11), S. 1179–1187. DOI: 10.1007/BF02540534.

Lomascolo, A.; Stentelaire, C.; Asther, M.; Lesage-Meessen, L. (1999): Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. In: *Trends Biotechnol.* 17 (7), S. 282–289. DOI: 10.1016/S0167-7799(99)01313-X.

Lowden, P. A. S.; Wilkinson, B.; Böhm, G. A.; Handa, S.; Floss, H. G.; Leadlay, P. F.; Staunton, J. (2001): Origin and True Nature of the Starter Unit for the Rapamycin Polyketide Synthase. In: *Angew. Chem.* 113 (4), S. 799–801. DOI: 10.1002/1521-3757(20010216)113:4<799::AID-ANGE7990>3.0.CO;2-5.

Malpartida, F.; Hopwood, D. A. (1984): Molecular cloning of the whole biosynthetic pathway of a Streptomyces antibiotic and its expression in a heterologous host. In: *Nature* 309 (5967), S. 462–464. DOI: 10.1038/309462a0.

Marsili, R. A. Y. (2016): Flavor, fragrance, and odor analysis. 2nd ed. Baco Raton: CRC Press.

Mayo, S. L.; Olafson, B. D.; Goddard, W. A. (1990): DREIDING. A generic force field for molecular simulations. In: *J. Phys. Chem.* 94 (26), S. 8897–8909. DOI: 10.1021/j100389a010.

McLafferty, F. W.; Tureček, F. (1995): Interpretation von Massenspektren. 3rd ed. Heidelberg, Berlin: Spektrum Akad. Verl.; Springer Spektrum.

Miller, D. J.; Allemann, R. K. (2012): Sesquiterpene synthases. Passive catalysts or active players? In: *Nat. Prod. Rep.* 29 (1), S. 60–71. DOI: 10.1039/c1np00060h.

Minerdi, D.; Bossi, S.; Gullino, M. L.; Garibaldi, A. (2009): Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. In: *Environ. Microbiol.* 11 (4), S. 844–854. DOI: 10.1111/j.1462-2920.2008.01805.x.

Minerdi, D.; Bossi, S.; Maffei, M. E.; Gullino, M. L.; Garibaldi, A. (2011): *Fusarium oxysporum* and its bacterial consortium promote lettuce growth and expansin A5 gene expression through microbial volatile organic compound (MVOC) emission. In: *FEMS Microbial. Ecol.* 76 (2), S. 342–351. DOI: 10.1111/j.1574-6941.2011. 01051.x.

Miyazawa, N.; Nakanishi, A.; Tomita, N.; Ohkubo, Y.; Maeda, T.; Fujita, A. (2009a): Novel key aroma components of galbanum oil. In: *J. Agric. Food Chem.* 57 (4), S. 1433–1439. DOI: 10.1021/jf803157j.

Miyazawa, N.; Tomita, N.; Kurobayashi, Y.; Nakanishi, A.; Ohkubo, Y.; Maeda, T.; Fujita, A. (2009b): Novel character impact compounds in Yuzu (*Citrus junos* Sieb. ex Tanaka) peel oil. In: *J. Agric. Food Chem.* 57 (5), S. 1990–1996. DOI: 10.1021/jf803257x.

Montero-Calderón, M.; Rojas-Graü, M. A. and Martín-Belloso O. (Hrsg.) (2010): Handbook of Fruit and Vegetable Flavors. Pineapple (*Ananas comosus* [L.] Merril) Flavor. 1st ed.: Hoboken, NJ, USA: John Wiley & Sons, Inc. Online verfügbar unter http://search.ebscohost. com/login. aspx?direct=true&scope=site&db= nlebk&db=nlabk&AN=330132.

Müller, R. (2004): Don't classify polyketide synthases. In: *Chem. Biol.* 11 (1), S. 4–6. DOI: 10.1016/j.chembiol.2004.01.005.

Nakanishi, K.; Watanabe, H. (2010): Fragrant enone compounds having natural, fresh fruit-like aroma and perfume/flavor compositions containing them. In: *Jpn Kokai Tokkyo Koho*. S. 22–27

National Institute of Standards and Technology: NIST chemistry web book. Online verfügbar unter http://webbook.nist.gov/chemistry, zuletzt geprüft am 30.08.2018.

Neiens, S. D.; Steinhaus, M. (2018): Odor-Active Compounds in the Special Flavor Hops Huell Melon and Polaris. In: *J. Agric. Food Chem.* 66 (6), S. 1452–1460. DOI: 10.1021/acs.jafc.7b05859.

Nuhn, P.; Wessjohann, L. (2006): Naturstoffchemie. Mikrobielle, pflanzliche und tierische Naturstoffe. 4th ed. Stuttgart: Hirzel.

Ogura, T.; Usuki, T. (2013): Total synthesis of acerogenins E, G and K, and centrolobol. In: *Tetrahedron* 69 (13), S. 2807–2815. DOI: 10.1016/j.tet.2013.01.065.

O'Hagan, D. (1991): The polyketide metabolites. 1st ed. New York: Ellis Horwood (Ellis Horwood series in organic chemistry).

Ohloff, G.; Pickenhagen, W.; Kraft, P. (2012): Scent and chemistry. The molecular world of odors. 2nd ed. Weinheim, Germany: Helvetica Chimica Acta; Wiley-VCH Verlag GmbH & Co. KG.

Oliynyk, M.; Stark, C. B. W.; Bhatt, A.; Jones, M. A.; Hughes-Thomas, Z. A.; Wilkinson, C. (2003): Analysis of the biosynthetic gene cluster for the polyether antibiotic monensin in *Streptomyces cinnamonensis* and evidence for the role of monB and monC genes in oxidative cyclization. In: *Mol. Microbial.* 49 (5), S. 1179–1190. DOI: 10.1046/j.1365-2958.2003.03571.x.

Orban, A.; Fraatz, M. A.; Rühl, M. (2019): Aroma Profile Analyses of Filamentous Fungi Cultivated on Solid Substrates. In: *Adv. Biochem. Eng. Biotechnol.* (169), S. 85–107. DOI: 10.1007/10_2019_87.

Ostermann, A. I.; Müller, M.; Willenberg, I.; Schebb, N. H. (2014): Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography – a comparison of different derivatization and extraction procedures. In: *Prostag. Leukotr. Ess.* 91 (6), S. 235–241. DOI: 10.1016/j.plefa.2014.10.002.

Palaniappan, N.; Kim, B. S.; Sekiyama, Y.; Osada, H.; Reynolds, K. A. (2003): Enhancement and selective production of phoslactomycin B, a protein phosphatase IIa inhibitor, through identification and engineering of the

corresponding biosynthetic gene cluster. In: *J. Biol. Chem.* 278 (37), S. 35552–35557. DOI: 10.1074/jbc.M305082200.

Pichersky, E.; Noel, J. P.; Dudareva, N. (2006): Biosynthesis of plant volatiles. Nature's diversity and ingenuity. In: *Science* 311 (5762), S. 808–811. DOI: 10.1126/science.1118510.

Pino, J. A. (2013): Odour-active compounds in pineapple (*Ananas comosus* [L.] Merril cv. Red Spanish). In: *Int. J. Food Sci. Tech.* 48 (3), S. 564–570. DOI: 10.1111/j.1365-2621.2012.03222.x.

Pleszczyńska, M.; Lemieszek, M. K.; Siwulski, M.; Wiater, A.; Rzeski, W.; Szczodrak, J. (2017): *Fomitopsis betulina* (formerly *Piptoporus betulinus*). The Iceman's polypore fungus with modern biotechnological potential. In: *World J. Microbiol. Biotechnol.* 33 (5), S. 83. DOI: 10.1007/s11274-017-2247-0.

Quin, M. B.; Flynn, C. M.; Schmidt-Dannert, C. (2014): Traversing the fungal terpenome. In: *Nat. Prod. Rep.* 31 (10), S. 1449–1473. DOI: 10.1039/c4np00075g.

Reis, F. S.; Pereira, E.; Barros, L.; Sousa, M. J.; Martins, A.; Ferreira, I. C. F. R. (2011): Biomolecule profiles in inedible wild mushrooms with antioxidant value. In: *Molecules* 16 (6), S. 4328–4338. DOI: 10.3390/molecules16064328.

Rohlfs, M.; Churchill, A. C. L. (2011): Fungal secondary metabolites as modulators of interactions with insects and other arthropods. In: *Fungal Genet. Biol.* 48 (1), S. 23–34. DOI: 10.1016/j.fgb.2010.08.008.

Sampedro, M. C.; Goicolea, M. A.; Unceta, N.; Sánchez-Ortega, A.; Barrio, R. J. (2009): Sequential stir bar extraction, thermal desorption and retention time locked GC-MS for determination of pesticides in water. In: *J. Sep. Sci.* 32 (20), S. 3449–3456. DOI: 10.1002/jssc.200900383.

Schimanski, S.; Krings, U.; Berger, R. G. (2013): From cabbage cuttings to pineapple flavor: Ready to use flavors from side streams of the food industry. In Hofmann T., Krautwurst D. und Schieberle P. (Hrsg.): Proceeding of Wartburg Symposium on Flavor Chemistry and Biology, S. 426–429.

Schimanski, S.; Krings, U.; Berger, R. G. (2013): Rapid analysis of volatiles in fat-containing matrices for monitoring bioprocesses. In: *Eur. Food Res. Technol.* 237 (5), S. 739–746. DOI: 10.1007/s00217-013-2047-1.

Schmidt-Dannert, C. (2015): Biosynthesis of terpenoid natural products in fungi. In: *Adv. Biochem. Eng./Biotechnol.* 148, S. 19–61. DOI: 10.1007/10_2014_283.

Schrader, J.; Bohlmann, J. (2015): Biotechnology of Isoprenoids. 1st ed. Berlin, Hedelberg: Springer International Publishing.

Schwab, W.; Fuchs, C.; Huang, F.-C. (2013): Transformation of terpenes into fine chemicals. In: *Eur. J. Lipid Sci. Technol.* 115 (1), S. 3–8. DOI: 10.1002/ejlt.201200157.

Schwenk, D.; Nett, M.; Dahse, H.-M.; Horn, U.; Blanchette, R. A.; Hoffmeister, D. (2014): Injury-induced biosynthesis of methyl-branched polyene pigments in a white-rotting basidiomycete. In: *J. Nat. Prod.* 77 (12), S. 2658–2663. DOI: 10.1021/np500552a.

Scott, D. B.; Jameson, G. B.; Parker, E. J. (2004): Isoprenoids: Gene Clusters and Chemical Puzzles. In: Tkacz J. S. und Lange L. (Hrsg.): Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine, Bd. 64. Boston, MA: Springer US; Imprint; Springer, S. 163–198.

Seshime, Y.; Juvvadi, P. R.; Fujii, I.; Kitamoto, K. (2005): Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*. In: *Biochem. Biophys. Res. Commun.* 331 (1), S. 253–260. DOI: 10.1016/j.bbrc.2005.03.160.

Shen, B. (2003): Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. In: *Curr. Opin. Chem. Biol.* 7 (2), S. 285–295. DOI: 10.1016/S1367-5931(03)00020-6.

Siebert, T. E.; Wood, C.; Elsey, G. M.; Pollnitz, A. P. (2008): Determination of rotundone, the pepper aroma impact compound, in grapes and wine. In: *J. Agric. Food Chem.* 56 (10), S. 3745–3748. DOI: 10.1021/jf800184t.

Solvay Deutschland (2019): Solvay hat das Vanillin, das die Lebensmittelindustrie heute benötigt. Online verfügbar unter https://www.solvay.de/artikel/solvay-hat-das-vanillin-das-die-lebensmittelindustrie-heute-benoetigt, zuletzt aktualisiert am 24.06.2019, zuletzt geprüft am 26.01.2019.

Sprecher, E. (1963): Über ätherisches Öl aus Pilzen. In: *Planta Med.* 11 (02), S. 119–127. DOI: 10.1055/s-0028-1100224.

Starks, C. M.; Back, K.; Chappell, J.; Noel, J. P. (1997): Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. In: *Science* 277 (5333), S. 1815–1820.

Staunton, J.; Caffrey, P.; Aparicio, J. F.; Roberts, G. A.; Bethell, S. S.; Leadlay, P. F. (1996): Evidence for a double-helical structure for modular polyketide synthases. In: *Nat. Struct. Boil.* 3 (2), S. 188–192. DOI: 10.1038/nsb0296-188.

Staunton, J.; Weissman, K. J. (2001): Polyketide biosynthesis. A millennium review. In: *Nat. Prod. Rep.* 18 (4), S. 380–416. DOI: 10.1039/a909079g.

Steele, C. L.; Crock, J.; Bohlmann, J.; Croteau, R. (1998): Sesquiterpene synthases from grand fir (Abies grandis). Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of delta-selinene synthase and gamma-humulene synthase. In: *J. Biol. Chem.* 273 (4), S. 2078–2089.

Steingass, C. B.; Carle, R.; Schmarr, H.-G. (2015): Ripening-dependent metabolic changes in the volatiles of pineapple (*Ananas comosus* (L.) Merr.) fruit. I. Characterization of pineapple aroma compounds by comprehensive two-dimensional gas chromatography-mass spectrometry. In: *Anal. Bioanal. Chem.* 407 (9), S. 2591–2608. DOI: 10.1007/s00216-015-8474-z.

Stevens, R. (1992): Dictionary of terpenoids. In: *Flavour Fragr. J.* 7 (4), S. 242–243. DOI: 10.1002/ffj.2730070418.

Studt, L.; Janevska, S.; Niehaus, E.-M.; Burkhardt, I.; Arndt, B.; Sieber, C. M. K. (2016): Two separate key enzymes and two pathway-specific transcription factors are involved in fusaric acid biosynthesis in *Fusarium fujikuroi*. In: *Environ. Microbiol.* 18 (3), S. 936–956. DOI: 10.1111/1462-2920.13150.

Swift, K. A. D. (Hrsg.) (1999): Current Topics in Flavours and Fragrances. Towards a New Millennium of Discovery. Dordrecht, s.l.: Springer Netherlands.

Takeoka, G.; Buttery, R. G.; Flath, R. A.; Teranishi, R.; Wheeler, E. L.; Wieczorek, R. L.; Guentert, M.: Volatile Constituents of Pineapple (*Ananas comosus* [L.] Merr.). In: Teranishi, R.; Buttery, R. G.; Shahidi, F. (Hrsg.): Flavor Chemistry: Trends and developments, Bd. 388, Washington: American Chemical Society S. 223–237.

Tkacz, J. S.; Lange, L. (Hrsg.) (2004): Advances in fungal biotechnology for industry, agriculture, and medicine. New York, NY: Kluwer Academic/Plenum Publishers. Online verfügbar unter http://www.loc.gov/catdir/enhancements/fy0819/2003060293-d.html.

Tokitomo, Y.; Seinhaus, M.; Büttner, A.; Schieberle, P. (2014): Odor-Active Constituents in Fresh Pineapple (*Ananas comosus* [L.] Merr.) by Quantitative and Sensory Evaluation. In: *Biosci., Biotechnol., Biochem.* 69 (7), S. 1323–1330. DOI: 10.1271/bbb.69.1323.

Tomita, N. (2010): Identification of Yuzunone and evaluation of its derivatives. In: *Nippon Koryo Kyokai Nr.* 248, S. 69–76.

Trapp, T.; Jäger, D. A.; Fraatz, M. A.; Zorn, H. (2018): Development and validation of a novel method for aroma dilution analysis by means of stir bar sorptive extraction. In: *Eur. Food Res. Technol.* 244 (5), S. 949–957. DOI: 10.1007/s00217-017-3003-2.

Unsicker, S. B.; Kunert, G.; Gershenzon, J. (2009): Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. In: *Curr. Opin. Plant Biol.* 12 (4), S. 479–485. DOI: 10.1016/j.pbi.2009.04.001.

van den Dool, H.; Kratz, P. (1963): A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. In: *J. Chrom. A* 11, S. 463–471. DOI: 10.1016/S0021-9673(01)80947-X.

Wallach, O. (1887): Zur Kenntnis der Terpene und ätherischen Oele. In: *Justus Liebigs Ann. Chem.* 238 (1-2), S. 78–89. DOI: 10.1002/jlac.18872380104.

Watanabe, K.; Praseuth, A. P.; Wang, C. C. C. (2007): A comprehensive and engaging overview of the type III family of polyketide synthases. In: *Curr. Opin. Chem. Biol.* 11 (3), S. 279–286. DOI: 10.1016/j.cbpa.2006.11.041.

Wedler, H. B.; Pemberton, R. P.; Tantillo, D. J. (2015): Carbocations and the Complex Flavor and Bouquet of Wine: Mechanistic Aspects of Terpene Biosynthesis in Wine Grapes. In: *Molecules* 20 (6), S. 10781–10792. DOI: 10.3390/molecules200610781.

Weissman, K. J. (2009): Chapter 1 Introduction to Polyketide Biosynthesis. In: D. A. Hopwood (Hg.): Complex Enzymes in Microbial Natural Product Biosynthesis, Part

B: Polyketides, Aminocoumarins and Carbohydrates, Bd. 459: Elsevier (Methods in Enzymology), S. 3–16.

Weissman, K. J.; Leadlay, P. F. (2005): Combinatorial biosynthesis of reduced polyketides. In: *Nature reviews. Microbiology* 3 (12), S. 925–936. DOI: 10.1038/nrmicro1287.

Yoshikuni, Y.; Ferrin, T. E.; Keasling, J. D. (2006): Designed divergent evolution of enzyme function. In: *Nature* 440 (7087), S. 1078–1082. DOI: 10.1038/nature 04607.

Zhang, W.; Tang, Y. (2008): Combinatorial biosynthesis of natural products. In: *J. Med. Chem.* 51 (9), S. 2629–2633. DOI: 10.1021/jm701269v.

Zheng, X.; Li, G.-H.; Xie, M.-J.; Wang, X.; Sun, R.; Lu, H.; Zhang, K.-Q. (2013): Stereumins K-P, sesquiterpenes from the fungus *Stereum* sp. CCTCC AF 2012007. In: *Phytochem.* 86, S. 144–150. DOI: 10.1016/j.phytochem.2012.10.014.

Ziegler, H. (Hrsg.) (2007): Flavourings. Production, composition, applications, regulations. 2nd ed. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KG. Online verfügbar unter http://deposit.dnb.de/cgi-bin/dokserv?id=2786957&prov= M&dok_var=1&dok_ ext=htm.

Lebenslauf

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Publikationsliste

Diese Arbeit basiert auf drei in *peer reviewed* Zeitschriften veröffentlichen Artikel.

Grosse, M.; Wu, S.; Krings, U. and Berger, R.G. (2020). *Formation of Decatrienones with a Pineapple-like Aroma from 1-^{13}C-Acetate by Cell Cultures of the Birch Polypore, Fomitopsis betulina*. J. Agric. Food Chem. DOI: 10.1021/acs.jafc. 9b07494.

Grosse, M.; Pendzialeck, T.; Fohrer, J.; Berger, R.G. and Krings, U. (2020). *(5E/Z,7E,9)-Decatrien-2-ones, Pineapple-like Flavors from Fomitopsis betulina - Structure Elucidation and Sensorial Properties*. J. Agric. Food Chem. DOI: 10.1021/ acs.jafc.9b06105.

Grosse, M.; Strauss, E.; Krings, U. and Berger, R.G. (2019). *Response of the sesquiterpene synthesis in submerged cultures of the Basidiomycete Tyromyces floriformis to the medium composition*. Mycologia 111, 885-894. DOI: 10.1080/ 00275514.2019.1668740.

Nachfolgend gelistete Veröffentlichungen sind im Rahmen dieser Doktorarbeit entstanden, jedoch nicht Teil dieser Dissertationsschrift.

Jorissen, T.; **Meyer, M**.; Detering, T.; Berger, R. G. und Recke, G. (2018): *Bioökonomische Analyse von Nebenströmen aus der Kartoffelverarbeitung unter Berücksichtigung einer biotechnologischen Verwertung, Austrian Journal of Agricultural Economics and Rural Studies*, Vol. 27.9. 61-68. DOI: 10.15203/ OEGA_27.9.

Prante, M.; Ude, C.; **Grosse, M**.; Raddatz, L.; Krings, U.; John, G.; Belkin, S. and Scheper, T. (2018). *A Portable Biosensor for 2,4-Dinitrotoluene Vapors, Sensors*, 18 (12), Nr. 4247. DOI: 10.3390/s18124247.

Es wurden Ergebnisse dieser Doktorarbeit auf folgenden Konferenzen vorgetragen:

Ersoy F.; **Grosse, M.**; Bohlke, C.; Zhang C.; and Berger, R. G. (2019). Enzymatic degradation of lignocellulosics. The Second Chinese-German Symposium “Functional and healthy food ingredients: Emerging insights and technologies”. 15. – 17.11. Wuxi, China

Grosse M.; Berger R. G. (2019). Biotechnological production of 5(*E/Z*),7(*E*),9-Decatrien-2-one. Young Scientists Conference “Food Biotechnology”, 23. – 25.09. Stuttgart, Deutschland

Meyer M.; Berger R. G. (2017). Influencing the sesquiterpen production of *Tyromyces floriformis* in submerged cultivation. Young Scientist Conference “Current Topics in Food Biotechnology”, 28. – 30.08. Rauischholzhausen, Deutschland

Weiterhin wurden Teilergebnisse der Doktorarbeit in folgenden Postern präsentiert:

Grosse, M.; Pendzialek, T.; Krings, U. and Berger, R. G. (2019). 5(*E*/*Z*),7(*E*),9-Decatrien-2-ones, Pineapple-like Flavour compounds from *Fomitopsis betulina* Structure Elucidation and Sensorial Properties. DECHEMA. 29.11., Frankfurt, Deutschland

Grosse, M.; Pendzialek, T.; Krings, U. and Berger, R. G. (2019). 5(*E*/*Z*),7(*E*),9-Decatrien-2-ones, Pineapple-like Flavour compounds from *Fomitopsis betulina* Structure Elucidation and Sensorial Properties. 12th Wartburg Symposium on Flavour Chemistry and Biology. 21. – 24.05., Eisenach, Deutschland

Meyer, M.; Krings, U. and Berger, R. G. (2018). Regulating Sesquiterpene Production of the Basidiomycete *Tyromyces floriformis* in Submerged Cultures. Chinese Symposium “functional and healthy food ingredients generated through state-of-the-art biotechnology”, 12. – 14.09., Stuttgart, Deutschland

Grosse, M.; Krahe, N.-K.; Rottmann, E.; Berger R. G. (2018). Cultivation of enzyme secreting higher fungi on side streams of the food processing industries, 9th International Congress on Biocatalysis, Biocat 2018, 30.8-3.9., Hamburg, Deutschland.

Meyer, M.; Krings, U. and Berger, R. G. (2018). Polysaccharide Impact on Sesquiterpene Production in Submerged Cultures of the Basidiomycete *Tyromyces floriformis*, Himmelfahrtstagung 07. – 09.05., Magdeburg, Deutschland