Technische Universität München

Fakultät für Chemie | Werner Siemens-Lehrstuhl für Synthetische Biotechnologie

Biosynthetic process strategies for the sustainable production of bioactive terpenoids in recombinant cell systems

WOLFGANG MISCHKO

Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

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Vorsitzender:		Prof. Dr. Tom Nilges
Prüfende der Dissertation:	1.	Prof. Dr. Thomas Brück
	2.	apl. Prof. Dr. Wolfgang Eisenreich
	3.	Prof. Dr. Robert Kourist

Die Dissertation wurde am 31.05.2019 bei der Technischen Universität München eingereicht und durch die promotionsführende Einrichtung Fakultät für Chemie am 30.09.2019 angenommen

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DANKE!

An erster Stelle möchte mich besonders bei meinem Doktorvater Prof. Dr. Thomas Brück für eine großartige Zeit am Werner Siemens-Lehrstuhl für Synthetische Biotechnologie bedanken. Er gab mir die Chance Teil seiner Arbeitsgruppe und der Entwicklung seines Lehrstuhls zu sein. Das entgegengebrachte Vertrauen ermöglichte mir sowohl eine fachliche, aber auch eine persönliche und menschliche Weiterentwicklung. Als mein Betreuer hat mich Dr. Norbert Mehlmer über die gesamte Zeit, und besonders zu Beginn, als noch alles neu und ungewohnt war, ausnahmslos und hilfsbereit unterstützt. Ich konnte immer von seiner Expertise und Erfahrung profitieren.

Keinesfalls wären die gesamten Herausforderungen ganz allein und ohne die Unterstützung kompetenter und hilfsbereiter Kollegen zu bewältigen gewesen. Sowohl beim Erstellen der Publikationen als Grundlage dieser Arbeit, als auch bei der Erhebung der dafür notwendigen Daten. Im Speziellen seien Dr. Monika Fuchs, Tom Schuffenhauer, Martina Haack, und Dr. Dominik Maslak für ihre Hilfsbereitschaft und ihren tatkräftigen Einsatz erwähnt. Selbstverständlich gilt mein Dank ebenso allen Studenten, welche ich über die Jahre betreuen durfte. Hoffentlich konnte ich euch auch das ein oder andere beibringen und die Faszination an unserem Forschungsgebiet vermitteln.

Der gesamten Arbeitsgruppe danke ich für eine herausragende Zeit, in welcher ich gute Freunde und Weggefährten fürs Leben finden durfte. Durch euch wurde diese Zeit zu einem besonderen Kapitel in meinem Leben, das ich auf keinen Fall vermissen möchte, und an welches ich immer gerne zurückdenke. Zusammen mit meinen Bürokollegen habe ich unzählige gewinnbringende und unterhaltsame, aber auch aufbauende und motivierende Stunden verbracht.

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Abstract

To preserve the environment and biodiversity in the face of a constantly growing population and the associated challenges, it is necessary to reconsider and modify our conventional industrial production routes. Specifically, with respect to agricultural plant protection, a dogmatic change towards sustainable, non-toxic solutions is required. Excessive use of predominantly synthetic chemicals for plant protection inevitably results in long-term adverse effects on insect biodiversity. The excessive use of chemical and highly toxic plant protectants also poses an immense risk to humans, who depend on pollination services for food production.

Overall, there is an increasing demand for renewable, natural product-based solutions in many areas of commodity products, including agrochemical, food, cosmetic and pharmaceutical applications. This drive for renewable solutions in almost all industrially relevant areas has led to numerous studies regarding the biosynthesis and biological activities of natural compounds. The current production of such substances is based on cost-intensive extraction from natural sources or conventional total chemical synthesis. However, limited by the structural complexity of many target components or insufficient product titer in the originating organism, these production methods often face technical or natural limitations. Simultaneously, the sustainability aspect of many production platforms is increasingly relevant in terms of resource efficiency and emerging waste streams. A particular goal of modern economy should be long-term independence from fossil resources as well as a responsible change to more sustainable global concepts. These have to encompass both the target molecule and the corresponding production route.

Ongoing progress in the field of synthetic biotechnology is providing promising renewable alternatives to conventional chemical products and their manufacturing processes. The transfer of genetic information from natural sources to industrially applicable production platforms enables the efficient and sustainable transformation of renewable resources. At the same time, waste materials can be converted into valuable enzymes and fine chemicals. However, the commercial biotechnological production of bioactive components is still severely underrepresented and behind its full potential. Reasons for this include the extensive efforts for industrial process development as well as the still pending establishment of suitable biotechnological tools and platforms. The functional characterization of productive enzymes for the efficient generation of desired target molecules is decisive. Both plants and especially fungi represent an enormous source of new and unexplored enzymes and routs of synthesis. The industrial

implementation of this biotechnological potential is currently still at the very beginning and will be developed progressively.

The scope of this work is especially focused on terpenes as a highly interesting industrial substance class with already diverse commercial applications. An essential goal was the development of a stable and efficient bacterial platform for the heterologous generation of diverse bioactive terpene structures. Further development of this system required the targeted modulation of relevant enzyme activities, the adaptation of operon structures and synthesis pathways, as well as an adequate supply of precursor molecules. The development of processes in the areas of fermentation, product recovery and purification was another priority. On this basis, the heterologous production of different terpene classes as well as the identification and characterization of new unknown terpene cyclases were realized.

This led to the establishment of a holistic and sustainable production process for the biosynthesis of a biological insecticide based on the diterpenoid cembratriene-ol. The conversion of industrial waste materials into this industrially valuable product was carried out utilizing a biotechnologically exploited terpene synthase of the tobacco plant. Various bioassays confirmed the bioactive spectrum of the produced components and offered additional antibacterial application possibilities. A stable fermentation process and the corresponding product extraction methods were developed for the launch into industrial implementation. The separation of the individual isomers required both purification by conventional chromatography methods and the incorporation of new liquid-liquid extraction-based techniques.

Furthermore, new and previously unknown enzymes of the Basidiomycota *Coniophora puteana* were characterized as sesquiterpene cyclases and their remarkable product spectra were identified. Due to their outstanding product selectivity, they are perfectly suited as novel biotechnological tools for the industrial production of the sesquiterpene cubebol, which is used in the food industry due to its refreshing character. Moreover, the characterization of these enzymes represents an important contribution in providing insight into the so far poorly investigated terpenome of fungi in reference to their biotechnological potential.

ZUSAMMENFASSUNG

Um in Anbetracht einer stetig anwachsenden Bevölkerung und den damit einhergehenden Herausforderungen Umwelt und Artenvielfalt zu bewahren, erfordert es ein Überdenken und Anpassen unserer konventionellen industriellen Produktionsrouten. Besonders im Bereich der Agrarwirtschaft sowie der damit verbundenen Verwendung von Agrochemikalien ist ein Umstieg auf nachhaltige Alternativen erforderlich. Der übermäßige Einsatz herkömmlicher, meist synthetischer Chemikalien hat unweigerlich langfristige negative Auswirkungen auf eine Vielzahl an Insekten und betroffene Ökosysteme. Dies birgt auch für den Menschen, dessen Nahrungsmmittelproduktion von deren Bestäubungsdiensten abhängig ist, ein enormes Risiko.

Zeitgleich steigt in beinahe allen industriell relevanten Bereichen die Nachfrage an Naturstoffen stetig an und hat zu zahlreichen Studien bezüglich deren Biosynthese geführt. Hinsichtlich dieser Tatsache stellt sich in Zukunft umso mehr die Frage nach einer ausreichenden, aber zeitgleich möglichst nachhaltigen Versorgung. Die aktuelle Produktion dieser Naturstoffe für die Anwendung in Lebensmitteltechnik, Agrochemie, Kosmetik und auch der Pharmazie basiert meist auf der kostenintensiven Extraktion aus natürlichen Quellen oder der konventionellen chemischen Totalsynthese. Eingeschränkt durch die strukturelle Komplexität vieler Zielkomponenten oder unzureichender Produkttiter im Ursprungsorganismus stoßen diese Produktionsmethoden jedoch oftmals an technische beziehungsweise auch natürliche Grenzen. Zeitgleich gewinnt der Nachhaltigkeitsaspekt vieler Produktionsplattformen immer mehr an Bedeutung in Bezug auf Ressourceneffizienz und resultierende Abfallströme. Ein Ziel modernen Wirtschaftens muss die langfristige Unabhängigkeit von fossilen Rohstoffen, so wie der verantwortungsvolle Wandel zu nachhaltigeren Gesamtkonzepten sein. Diese müssen sowohl das Endprodukt als auch die entsprechende Produktionsroute vollumfänglich berücksichtigen.

Durch einen immer schnelleren Fortschritt im Bereich der synthetischen Biotechnologie eröffnen sich vielversprechende Alternativen zu konventionellen chemischen Einsatzstoffen und deren Herstellungsprozessen. Die Übertragung genetischer Information aus natürlichen Quellen in industriell nutzbare Produktionsplattformen ermöglicht einerseits die effiziente und nachhaltige Transformation erneuerbarer Ressourcen. Andererseits können auch überschüssige Reststoffe in wertvolle Enzyme und Feinchemikalien umgewandelt werden. Bis zum heutigen Tag ist die kommerzielle biotechnologische Produktion von bioaktiven Komponenten allerdings noch stark

unterrepräsentiert und bleibt hinter ihrem Potenzial zurück. Gründe dafür sind der zum Teil immer noch hohe Aufwand für die industrielle Prozessentwicklung, als auch die oft noch ausstehende Etablierung passender biotechnologischer Werkzeuge und Plattformen. Im Fokus steht hier die funktionelle Charakterisierung potenter Enzyme für die effiziente katalytische Umwandlung hin zum gewünschten Zielmolekül. In diesem Zusammenhang stellen sowohl Pflanzen, aber im Besonderen auch das Reich der Pilze eine enorme Quelle neuer und unerforschter Biokatalysatoren und Synthesewege dar. Die industrielle Implementierung dieses biotechnologischen Potenzials steht gegenwärtig noch am Anfang und wird erst schrittweise erschlossen.

Der Fokus dieser Arbeit liegt im Speziellen auf den Terpenen als eine industriell hoch interessante Stoffklasse mit bereits vielfältigen Anwendungen im kommerziellen Bereich. Ein elementares Ziel war die Entwicklung einer stabilen und zugleich effizienten bakteriellen Plattform für die heterologe Erzeugung vielfältiger bioaktiver Terpenstrukturen. Die kontinuierliche Weiterentwicklung dieses Systems erforderte die gezielte Modulation spezifischer Enzymaktivitäten, die Anpassung der Operon-Strukturen und Synthesewege, sowie die ausreichende Versorgung an Vorläufermolekülen. Des Weiteren stand die Prozessentwicklung im Bereich Fermentation, Produktisolation und Aufreinigung im Fokus. Basierend auf dieser Grundlage wurde die heterologe Produktion verschiedener Terpenklassen sowie die Identifizierung und Charakterisierung neuer, bisher unbekannter Terpensynthasen erfolgreich umgesetzt.

Daraus ging die Etablierung eines ganzheitlichen und nachhaltigen Produktionsprozesses für die Herstellung eines biologischen Insektizids auf Basis des Diterpens Cembratriene-ol hervor. Die Umwandlung von industriellen Abfallstoffen in dieses industriell wertvolle Produkt erfolgte mit Hilfe einer biotechnologisch erschlossenen Terpensynthase aus der Tabakpflanze. Diverse Bioassays bestätigten das bioaktive Spektrum der produzierten Komponenten und eröffneten zusätzlich neben dem Einsatz als Bioinsektizid auch antibakterielle Anwendungsmöglichkeiten. Für den Einstieg in die industrielle Umsetzung wurden ein stabiler Fermentationsprozess und die entsprechenden Produkt-Extraktionsmethoden entwickelt. Die Aufschlüsselung der einzelnen Isomere erforderte dafür sowohl die Aufreinigung mittels konventioneller Chromatografie, als auch das Einbeziehen moderner Flüssig-Flüssig-Extraktion basierter Techniken.

Des Weiteren wurden neue und bisher unbekannte Enzyme des Basidiomyceten *Coniophora puteana* als Sesquiterpencyclasen identifiziert, sowie ihr außergewöhnliches Produktspektrum charakterisiert. Durch die bisher einzigartig hohe Produktselektivität

eignen sich diese unter anderem hervorragend als neue biotechnologische Werkzeuge für die industrielle Produktion des Sesquiterpens Cubebol, welches aufgrund seines erfrischenden Charakters in der Lebensmittelindustrie eingesetzt wird. Die Charakterisierung dieser Enzyme stellt einen wichtigen Beitrag zum Einblick in das bisher nur schwach untersuchte Terpenom der Pilze hinsichtlich deren biotechnologischen Potenzials dar.

Parts of this thesis have been published in peer-reviewed journals:

Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents

Wolfgang Mischko, Max Hirte, Simon Roehrer, Hannes Engelhardt, Norbert Mehlmer, Mirjana Minceva and Thomas Brück

Green Chemistry (2018) 20, 2637–2650

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From microbial upcycling to biology-oriented synthesis: combining whole-cell production and chemo-enzymatic functionalization for sustainable taxanoid delivery

M. Hirte, W. Mischko, K. Kemper, S. Röhrer, C. Huber, M. Fuchs, W. Eisenreich, M. Minceva and T. B. Brück

Green Chemistry (2018) 20, 5374-5384

http://dx.doi.org/10.1039/C8GC03126F

Identification of sesquiterpene synthases from the Basidiomycota *Coniophora puteana* for the efficient and highly selective β-copaene and cubebol production in *E. coli*

Wolfgang Mischko, Max Hirte, Monika Fuchs, Norbert Mehlmer and Thomas B. Brück

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Poster presentation

Industrial Biotechnology Forum (IBF) 2016

Technische Universität München

14th - 15th March 2016, Garching bei München, Germany

Poster presentation

XV Fall workshop CICY 2016 "Frontiers in Biotechnology"

Yucatan Center for Scientific Research / Centro de Investigación Científica de Yucatán (CICY)

Mérida, Yucatán, Mexico

24th - 28th October 2016

Poster presentation

There is in fact no distinction between the fate of the land and the fate of people.
When one is abused, the other suffers.

- Wendell Berry

CONTENTS

CHAPTER I

SCIENTIFIC BACKGROUND

1 INTRODUCTION

1.1. THE NEED OF PIONEERING INNOVATIONS FOR A SUSTAINABLE FUTURE

Ecological and social responsibility is more important than ever. A constantly growing population, limited fossil resources and the undeniable global warming challenge our society profoundly.[1–3] Although the global agricultural food production has kept pace with the increasing population growth, prevalence of undernutrition still exists, concerning more than 820 million people with insufficient access to food.[4] Moreover, many environmental systems and processes are pushed to their limits by a worldwide development based on economic efficiency. Strong evidence indicates that food production has a huge impact on global environmental change, mainly due to deforestation and burning of biomass.[5] In this context, modern agriculture covers about 40% of global land,[6] is responsible for up to 30% of our greenhouse-gas emissions[7] and consumes 70% of the global freshwater demand.[8, 9] Besides different other human influences, conversion of previous natural ecosystems to agricultural farmland and the excessive use of chemicals to increase crop yields are the most serious factors for the advancing extinction of species and reduced biodiversity.[10–14] Recent studies show that 40% of the world's insect species decline in population and one third are threatened with extinction.[14] The total insect biomass has reduced by 75% within the last 30 years,[15] severely affecting biodiversity's capacity to support food production, gene flow, and other important ecosystem services.

Nevertheless, ensured food supply for the increasing number of people is a declared goal of our modern society, facing unpredictable future climate conditions[16, 17] and constantly decreasing fertile areas.[18] While in many regions of Europe, Russia, and North America, the net area for food production decreases, agricultural land has expanded considerably in the highly biodiverse tropics as a result of massive deforestation.[19] This alarming progress and the associated challenges demand a more sustainable and bio-based economy, not only for the present but also for future generations. Global agriculture is particularly affected, not only as the basis for human food supply and with the responsibility to preserve the environment, but also as a massive and diverse economic sector to meet the needs of an increasing population.[1, 12, 20, 21] Innovative solutions as well as a continuous optimization of yield efficiency are urgently required.

Even today, securing sustainable crop yields and global food requirements can only be guaranteed through the targeted use of agrochemicals.[22, 23] Synthetic products have been developed to support farmers worldwide in many aspects. The control of pathogens, weeds and insects that negatively affect plant productivity remains an

essential aspect of successful and economical large-scale food production. These types of agrochemicals especially include fertilizers, pesticides, hormones and plant growth regulators, which are intended to promote plant protection, improved yield and persistent plant growth.[24, 25] It is assumed that without active crop protection, global yields of important agricultural crops such as rice, wheat and barley would decline by about 50%.[18, 26] The enormous importance of the agrochemical industry is also reflected in the estimated global volume of sales of about $220 billion (2016).[27] At the current time, the global supply of agrochemicals is dominated by only a few major suppliers (DuPont, Syngenta, BASF, Dow AgroSciences and Bayer CropScience) covering 70-75% of the market.[18]

Insecticides in particular account for about 10% of the total demand for agrochemicals.[28, 29] However, their economic importance could increase significantly over the next few years, as insect pests might reproduce significantly better under changing climatic conditions, which could lead to increased crop losses.[30, 31] In today's global application of chemical insecticides, synthesized neonicotinoids have the largest market share. The first neonicotinoid-based insecticide, imidacloprid, was launched in 1991. Today, this class comprises at least seven main active ingredients with a market share of more than 25% of total insecticide sales worldwide.[32] Blockbuster products like imidacloprid, thiamethoxam and clothianidin combined accounted for 85% of the total neonicotinoid sales in 2014.[33] The fast-acting effect of these compounds in insects is mediated by neurotoxic activity through the irreversibly binding to nicotinic acetylcholine receptors.[34–36] As neurotoxins with systemic activity and high toxicity to most arthropods, they are intended to provide effective pest control and have been used extensively in all areas of farming, landscaping or private gardening. However, the mode of action is not limited to pest insects, but can also negatively affect a broad range of small animals and beneficial insects.[36, 37]

Even though a variety of insect pests are still efficiently controlled by neonicotinoids, their prevalence has led to an increasing selection pressure and the gradual emergence of natural resistances. In some species, this has already reached a level that significantly reduces the efficacy of these synthetic insecticides.[32] In addition, the neonicotinoid product class has recently attracted widespread attention as it poses potential hazards to the viability of entire ecosystems.[38]

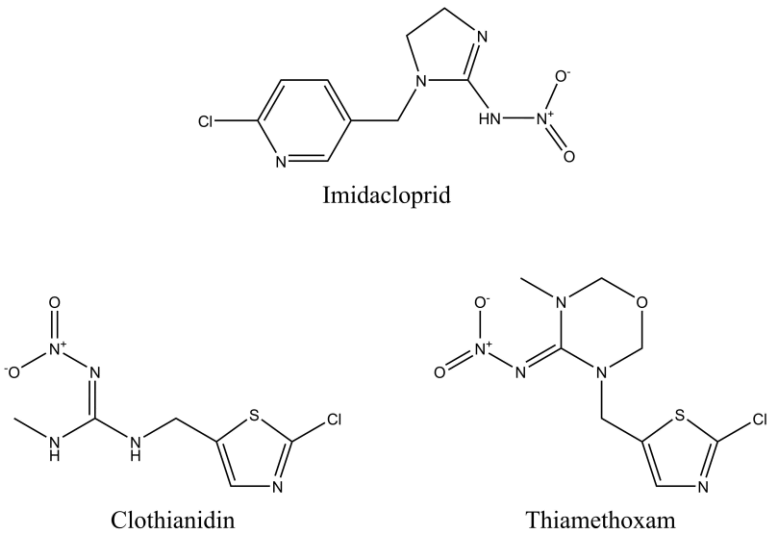

Figure 1: *The blockbuster compounds imidacloprid, thiamethoxam and clothianidin accounted for 85% of the total neonicotinoid insecticide sales in 2014. The application has been strictly limited since the European Union classified them as most hazardous neonicotinoids for bees with broad environmental effects.*

Recent comprehensive scientific research has clearly established a correlation between the continuously increased use of synthetic insecticides and a reduced biodiversity in flora and fauna. The extensive use needed for insect-management and a poor biodegradability lead to an accumulation in soil and groundwater over a long-term period. This also impairs adjacent terrestrial and aquatic ecosystems as well as non-target organisms such as pollinating bees, wild insects and soil invertebrates.[36–40] Insecticidal residues are found in nectar and pollen of treated plants, affecting generations of crops and the insects that depend on them. Even sub lethal doses are found to disturb capacity and behavior of bees and cause disorientation.[41] Queen bees influenced by insecticide residues, laying a significantly reduced number of eggs, are a direct risk for the survival of affected insect colonies.[42] The accumulation of toxic synthetic insecticides and their degradation products in soil and groundwater therefore presents a serious threat to agriculture itself and the survival of various ecosystems.[20, 38, 39]

Within the last years, the need for strict risk assessments to protect biodiversity and vulnerable ecosystems has been brought back into the focus of research and has led to concerns about global food security. An EFSA (European Food Safety Authority) study officially stated that bees are exposed to hazardous pesticides from pollen and nectar from fields treated with neonicotinoids.[43] It is also considered that neonicotinoids can be highly toxic to all kinds of insects as they cause paralysis and death by central nervous system disorders. The authority considered more than 1,500 studies for data

collection, including all relevant published literature. Due to this extensive environmental impact, and in order to protect bee health, biodiversity and food production, the European Union has decided to impose stricter limits on the use of neonicotinoid insecticides for all outdoor crops. As a result, the application of the three blockbuster neonicotinoids, classified as the most hazardous to bees' health - clothianidin, imidacloprid and thiamethoxam - has been limited to the use in permanent greenhouses only.[44, 45] However, a complete prohibition by itself will certainly not resolve the emerged problems. Farmers will inevitably switch to other methods and agents that include the next generation of synthetic pesticides. A permanent reduced distribution of this synthetic product class and a future sufficient replacement must be a declared goal of sustainable and modern agricultural principles. With regard to biodegradability and environmental impacts, a change of production methods, especially in the chemical industry is essential. New approaches for highly effective and highly specific insecticides with improved environmental safety as well as reduced toxicity to humans and animals must be the goal of future research and development.

Current alternatives to chemically synthesized insecticides are applications of bacterial systems[46, 47] or plant-based insecticides.[48] These combine several advantages, such as improved biodegradability by sunlight and microbes as well as their advanced and proven spectrum of biological activities against a variety of potential pests. Furthermore, they usually show higher target specificity and therefore lower toxicity to humans and the environment.[47–49] However, the large-scale use of products based on plant extracts is commonly limited by reduced availability, poor stability or inconsistent and globally varying product quality.[50, 51] As part of the secondary metabolism, these compounds occur usually in-planta only in low concentrations and as a mixture of various isoforms.[52–54] As a result, purification from renewable sources is in many cases technically demanding, expensive and only scalable to a limited extent, which severely restricts commercialization.[55, 56] However, the current developments in synthetic biology and bioprocess engineering offer focused production routes to tailored bioactive compounds as a sustainable alternative to chemical synthesis pathways for structurally complex compounds.[57, 58] A scalable and controlled production for an economic supply of bio-based insecticides by individual cell factories is possible.

1.2. Research project "Sustainable production of new bio-insecticides"

The project "Sustainable production of new bio-insecticides" was based on an economically and ecologically optimized concept for the biotechnological conversion of biogenic residues into a highly specific nature-identical bio-insecticide. As a main principle of this project, a tailor-made *E. coli* whole cell bio catalysis platform was to be generated, investigated and optimized. The primary vision is the substitution of biodegradable compounds for synthetic insecticides. The long-term shift to biological insecticides represents a major change in crop protection strategies that would support and enhance sustainable agricultural practices. The focus is on the protection of useful and endangered insect populations, which are essential for crop pollination.

Cembranoids represent a subgroup of the chemical class of terpenes. These specific compounds are a very promising class of bioactive substances and are characterized by simplified biosynthesis. In contrast to commercial insecticides, cembratriene-ol (CBT-ol), which was targeted in the present research project, has no nitrogen- or phosphate-containing nor halogenated structural elements. This leads to improved biodegradability under natural conditions.[59–62] In nature, they are mainly found in species of the tobacco plant (e.g.: *Nicotiana sylvestris* or *Nicotiana tabacum*).[52, 63] Their insecticidal effect has been demonstrated,[52] but their extensive characterization or industrial application was still lacking. The stereochemical complexity of the cembrane backbone prevents the efficient and sustainable production of cembranoid-based insecticides by chemical total synthesis.[64, 65] Furthermore, a controlled industrial supply of cembranoids by extraction from natural plant sources is not feasible with regard to quality and sustainability. Since cembranoids are also products of the secondary metabolism, they are only present in low concentrations in the natural production organism,[52] which significantly limits the economic efficiency of such a process. An industrially relevant production scale-up is therefore barely possible. However, modern biotechnological methods are capable of realizing cembranoid biosynthesis on a microbial basis.

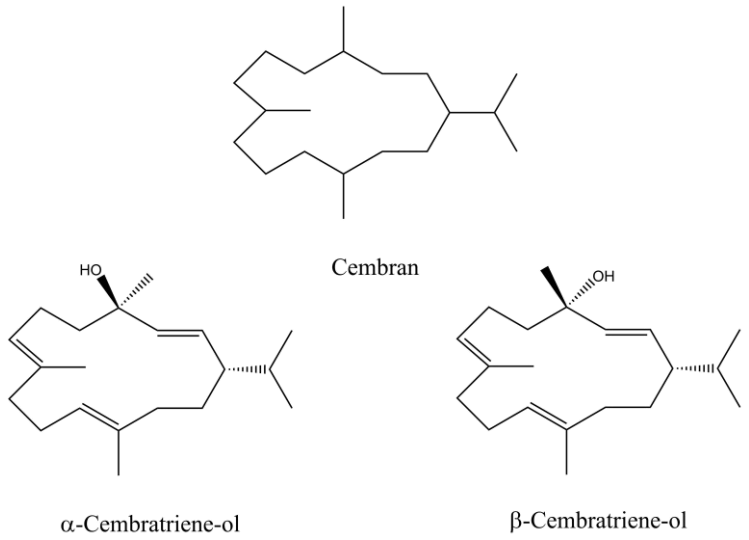

Figure 2: *Cembranoids represent diterpenes with a typical macrocyclic C14 backbone. In nature, cembratriene-ol (CBT-ol) is found as component of the cuticular wax or exudates of many tobacco plants and is part of the defense strategy against insects.*[52, 63] *Without any nitrogen-, phosphate- or halogenated elements, biodegradability is significantly improved compared to commercial insecticides.*[59–63, 66]

The transfer of the essential genetic information from the natural host into an industrially established microbial production strain facilitates scalable production under constantly controlled reaction conditions. By iterative process engineering in combination with continuous strain optimization, a process should be designed that enables an economical, bio-based compound production. Although wild type *E. coli* strains are naturally not efficient terpene producers, central precursors can be synthesized via their natural 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. By using biotechnological methods, the optimization of the *E. coli* metabolism network for the efficient production of a large number of effective terpenes can be achieved.[67, 68]

The fermentative production of cembranoids targeted in this research project relies on the use of biomass as a raw material basis in order to create an economically and ecologically optimized value chain. A preferred raw material for this purpose is wheat bran, a residue from cereal processing. About 90 million tons are produced worldwide per year.[69] Wheat bran contains a high proportion of poorly accessible hemicellulose and is therefore only used to a limited extent as an additive for animal feed and bakeries.[70] However, a large constituent (~60% w/w) consists of sugar polymers,[71] which can be enzymatically converted into fermentable monomeric sugars.[72] In contrast to previous approaches, the fermentative conversion of wheat bran hydrolysate into high-priced insecticides offers a closed, economically and ecologically optimized value chain. The sustainable production of modern bio-insecticides without large amounts of toxic

intermediates and excessive waste streams is expected to contribute significantly to a forward-looking agricultural practice that can meet the challenges of climate change and a constantly growing population.

The entire project was funded by the Bavarian Ministry of Economic Affairs, Energy and instructed by Prof. Thomas Brück from the Werner Siemens-Chair of Synthetic Biotechnology at the TU Munich. As a result the publication "Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents" was published.[66]

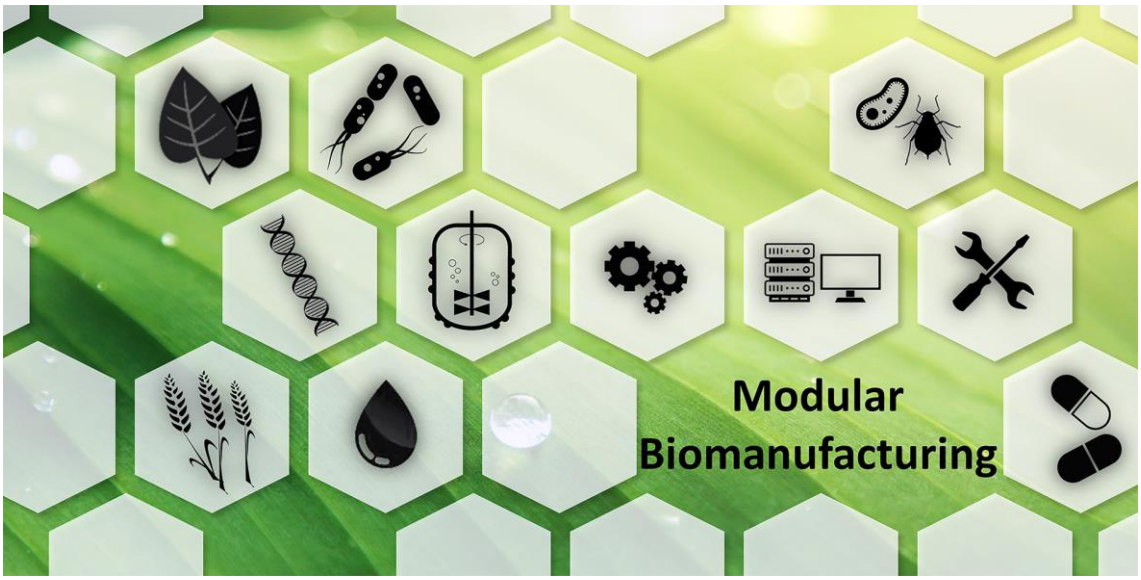

Figure 3: *The funded project "Sustainable production of new bio-insecticides" resulted in the publication "Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents" discussing the development of a biotechnological multi-step process for the production of a bio-based terpenoid insecticide.[66]*

1.3. THE DIVERSE CLASS OF TERPENES

Terpenes, also known as terpenoids or isoprenoids, comprise the most chemically and structurally diverse group of natural products.[73, 74] According to present numbers, this family with more than 80,000 characterized members, also including steroids and carotenoids, accounts for nearly one-third of all currently characterized natural compounds.[73] Almost all living organisms produce terpenes for certain essential physiological functions. However, the biological roles of the vast majority are still unknown and the functional characterization is lagging behind.[75]

Nevertheless, the scientific and economic interest in these natural compounds and their important biological functions is steadily growing. Especially as the primary constituents of essential oils, terpenes have already been identified and used for different areas of application.[76] Meanwhile, they have become important ingredients for the pharmaceutical,[77] cosmetic,[78] food[79] or fragrance[80] industry, and more recently, for advanced biofuels.[81] Since they are usually found as secondary metabolites in in very low abundance in many plants or naturally occurring microorganisms,[52–54, 75] there has been strong interest in the development of biotechnological production platforms. Different research teams have successfully engineered the isoprenoid biosynthetic pathways as basic requirement for the generation of valuable chemicals in microorganisms.[56]

Essentially, all terpenoids are based on relatively simple biosynthetic principles and originate from the same basic C5 isoprene building blocks, isopentenyl pyrophosphate (IPP) and the corresponding isomer dimethylallyl pyrophosphate (DMAPP). Head-to-tail connection of an IPP and DMAPP molecule is catalyzed by a geranyl pyrophosphate synthase (GPPS) according to the biogenic isoprene rule.[82] The resulting geranyl pyrophosphate (GPP) is the universal precursor for all monoterpenes. Cis-addition of further IPP-subunits by farnesyl pyrophosphate synthases (FPPSs) and geranylgeranyl pyrophosphate synthases (GGPPSs) generates the respective linear precursors for complex sesquiterpenes (farnesyl pyrophosphate, FPP) and diterpenes (geranylgeranyl pyrophosphate, GGPP). Further coupling generates even longer precursors molecules for sester- or triterpenes such as sterols and carotenoids.[73, 83–87]

The structural diversity within the class of terpenes arises from unique cyclization reactions of the linear precursors, which yield an extraordinary range of different natural products. These highly specific biochemical cascades are catalyzed by enzymes known as terpene synthases (TPSs) or, more accurate, terpene cyclases and constitute one of the most complex reactions to be found in nature. Multiple highly reactive intermediates are efficiently managed within the active site cavity of the TPS, allowing for a targeted modification of the substrate. Specialized enzymes, including cytochrome P450 monooxygenases (CYP) or methyltransferases add functional groups at different positions, further enhancing structural diversity and the spectrum of biological activity.[73, 88]

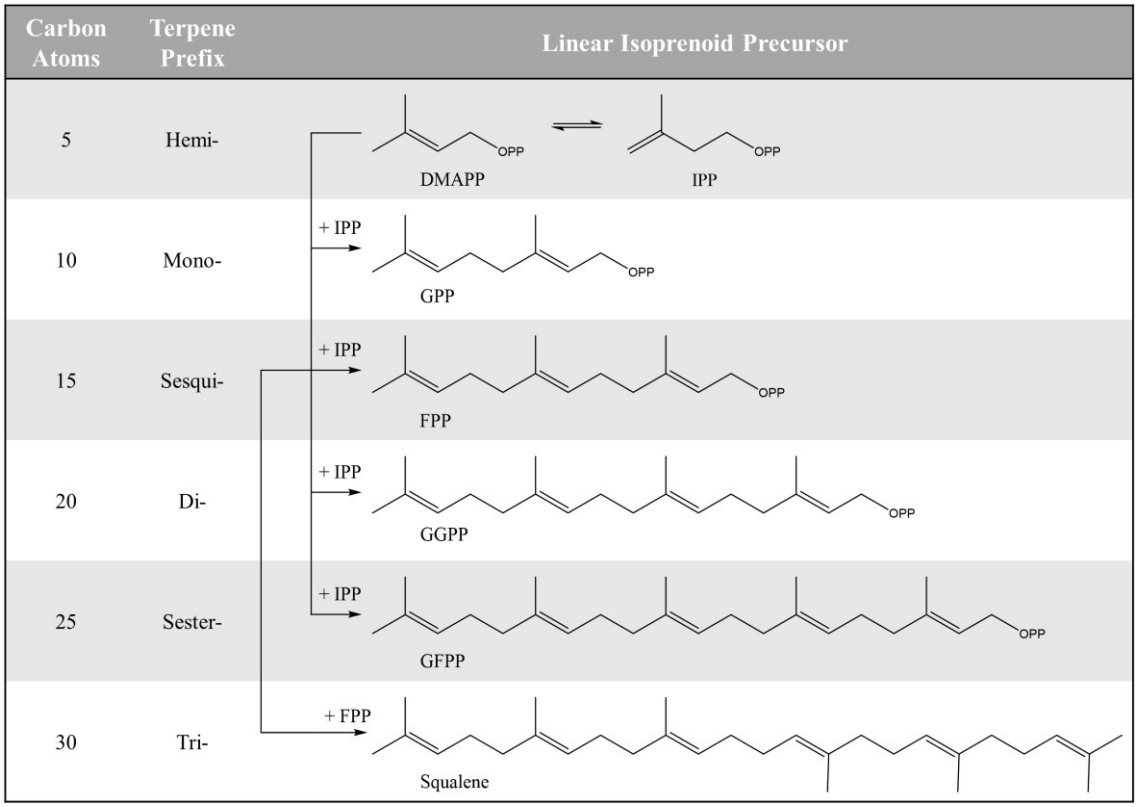

Figure 4: *General scheme of terpene nomenclature and the involved linear precursors. The structural diversity of terpenes is based on the coupling of basic C5 isoprene building blocks, isopentenyl pyrophosphate (IPP) and the corresponding isomer dimethylallyl pyrophosphate (DMAPP). Subsequent cyclization reactions yield a variety of different natural compounds.*[73]

Two independent pathways ensure the biological supply of IPP and DMAPP. Within the mevalonate (MVA) pathway isoprenoids originate from acetyl-coenzyme A.[89] Isoprenoids derived from the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway are based on the connection of glyceraldehyde 3-phosphate (G3P) and pyruvate.[90, 91] Both pathways require ATP and NADPH for the performance of the essential steps to produce IPP and DMAPP. With a few exceptions,[92] the cytosolic MVA pathway is primarily utilized by eukaryotes as well as Archaea,[93] while the MEP pathway is mainly present in most bacteria and in the plastids of all phototrophic organisms.[91, 94] Plants provide both pathways.[91, 95, 96]

Based on the relevant research areas and the generated results, the focus of this thesis lies on the sesqui- and di-terpenes. Therefore, the following section discusses their specific biosynthesis and the responsible enzymes in more detail.

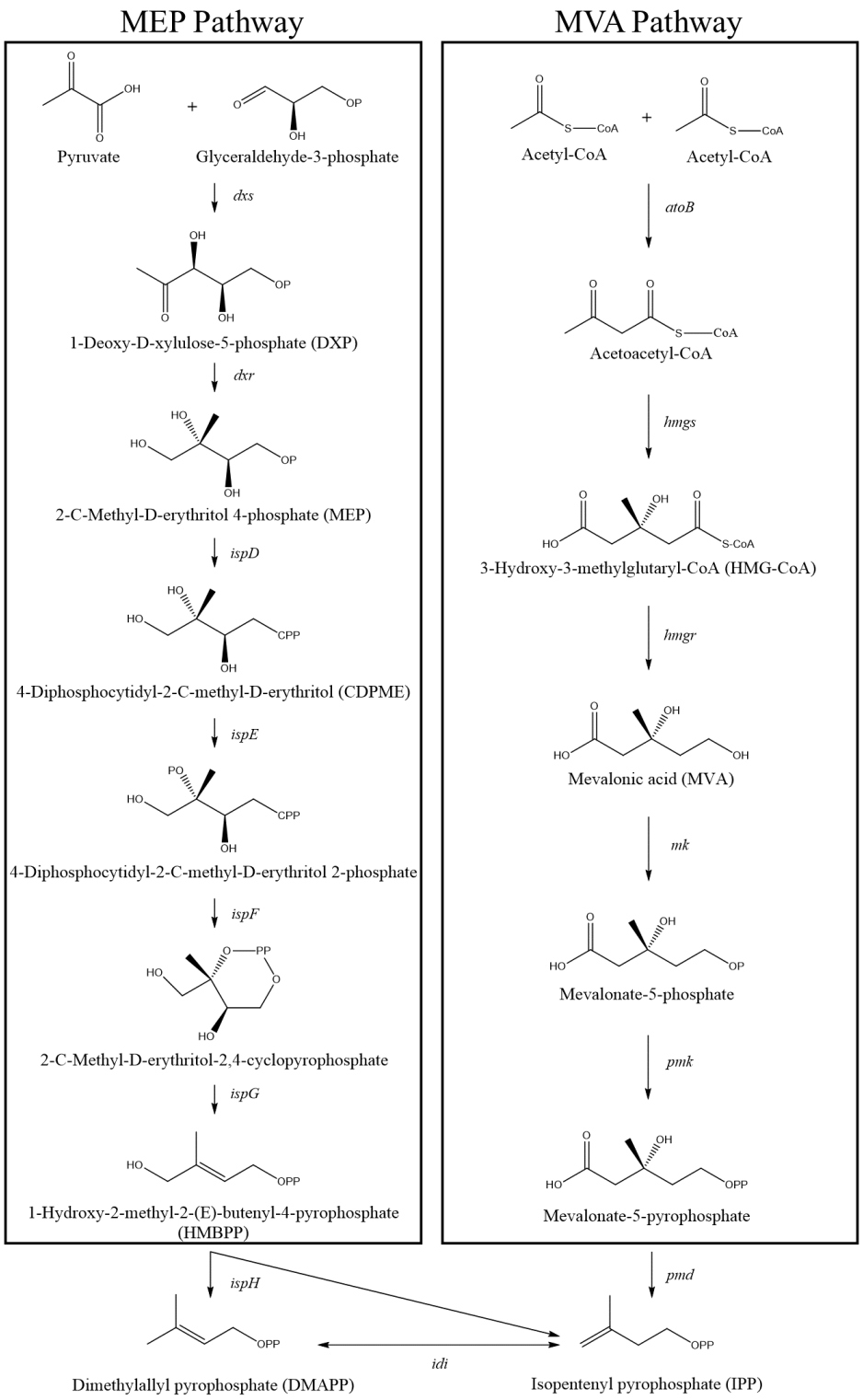

Figure 5: *Two biosynthetic pathways for terpenoid precursor supply exist in nature. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway consists of seven enzymatic steps and starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to generate IPP and DMAPP in a ratio of 5:1. The mevalonate (MVA) pathway transforms two molecules of acetyl-CoA within six enzymatic steps to IPP. IPP isomerase (idi) is required for converting IPP and DMAPP according to the respective demands.*[97] *Abbreviations: dxs: DXP synthase - dxr: DXP reductase - ispD: CDPME synthase - ispE: CDPME kinase - ispF: 2-C-Methyl-D-erythritol-2,4-diphosphate synthase - ispG: HMBPP synthase - ispH: HMBPP reductase - atoB: Acetoacetyl-CoA thiolase - hmgs: HMG-CoA synthase - hmgr: HMG-CoA reductase - mk: Mevalonate kinase - pmk: Phosphomevalonate kinase - pmd: Phosphomevalonate decarboxylase - idi: IPP isomerase*

1.3.1. Cyclization of FPP to generate the manifold sesquiterpene scaffold

Sesquiterpenoids represent a highly diverse group of different cyclic hydrocarbons, with over 13,900 described compounds[74] and more than 300 structural scaffolds.[98, 99] This family encompasses different industrial relevant compounds like (+)-nootkatone, which is considered to be a high-value compound due to its application as flavor[80, 100] or potential biopesticide and insect repellent.[101] Another industrial relevant candidate is artemisinin from the chinese medicinal herb *Artemisia annua*, as the active ingredient in therapies to treat malaria.[102, 103]

All sesquiterpenoids have in common their basic C15 backbone derived from the linear precursor FPP, which consists of three isoprene subunits.[85, 86] The linear FPP molecule is typically cyclized by specific metal-dependent class I sesquiterpene synthases (STPSs), to form complex hydrocarbon products containing one or more fused rings and stereocenters.[73] Characteristic features of class I TPSs are specific signature amino acid sequence motifs, which are indispensable for the enzymatic functionality. Typically, an aspartate-rich DDXXD motif and almost always a (N,D)D(L,I,V)X(S,T)XXXE, accordingly designated as the "NSE" motif, are located within the active site cavity. These amino acid residues are decisive for coordination of catalytically essential divalent Mg2+ metal ions, which stabilize the FPP associated pyrophosphate group within the active site cavity.[104–107] The initial step in the enzymatic cyclization reaction is a metal ion-induced ionization of the substrate and cleavage of the pyrophosphate. This promotes structural relocations and the sealing of the active site by the enzyme lid.[104] Subsequently, the resulting highly reactive carbocation intermediate usually undergoes an initial ring closure at position 1,10 or 1,11 followed by further bond forming cyclization reactions, hydride transfers, or methyl migrations.[73, 108] Complex ring rearrangements and biochemical modifications are stabilized by the aromatic residues lining the active site cavity of all TPSs, ensuring that the substrate and subsequently generated intermediates adopt only those conformations, which lead to the required product formation.[73] Finally, the reaction cascade is terminated either by the attack of a water molecule,[109–111] or by deprotonation.[112, 113] Products are released by the opening enzyme lid, which encloses the active site during the whole reaction. The basic scaffolds are often further decorated by CYPs or other modifying enzymes, thereby increasing the structural diversity.[73, 114]

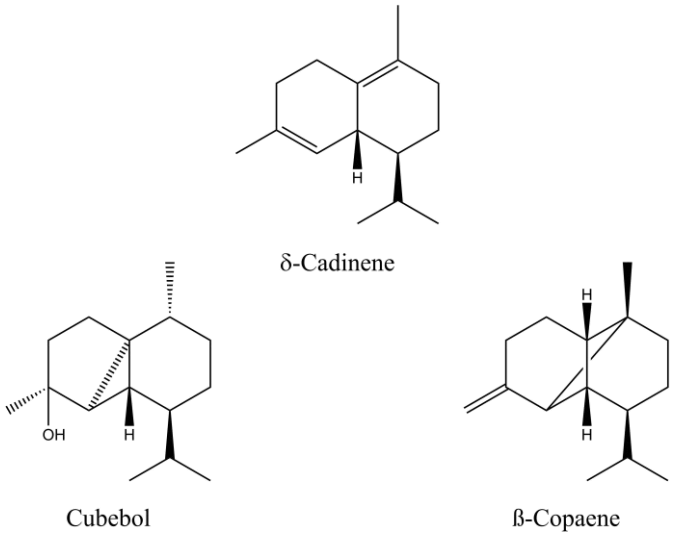

Figure 6: *There are several important sesquiterpenoids with different biological effects. Of particular importance for this thesis are cadalane and cubebane-type compounds. The tricyclic sesquiterpene cubebol is of interest to the cosmetic and flavor industry with a pronounced cooling effect for approved dietary supplements and flavoring agents. β-Copaene has so far received very limited attention. However, isoforms of this compound have already been associated with anticarcinogenic as well as antioxidant activities. Only few characterized STPSs provide the enzymatic formation of cubebol or β-copaene. The recent characterization of novel highly selective enzymes facilitates an efficient biotechnological production.*[115]

1.3.2. Cyclization of GGPP to produce the complex diterpene scaffold

Diterpenoids are with at least 12,500 described compounds[74] another highly diverse class of natural products. All diterpenoids are derived from the C20 precursor GGPP, which is composed of four isoprene units. The vast majority of diterpenoids is found in plants, but only for a very small fraction the physiological roles and functions are completely investigated.[116] Compared to smaller terpenes, the larger size of diterpenes reduces their volatility and shifts the potential range of biological functions. As products from secondary metabolism, they are usually not required for normal growth or development. Nevertheless, they commonly play an important biological role in attraction, resistance, defense or communication interactions.[75] Besides the extensive versatility in nature, the family of diterpenoids encompasses several high value compounds for human industry such as the tumor therapeutic blockbuster paclitaxel[117, 118] or the fragrance precursor sclareol.[119]

Biosynthetic pathways for diterpene generation and cyclization require a dedicated GGPPS, extending the previous sesquiterpene precursor FPP by another IPP subunit.[120] Starting from the resulting linear GGPP molecule, diterpene scaffold formation occurs likewise via a carbocation cascade. In contrast to STPSs, an additional enzyme class II

exists for diterpene cyclization, with a separate essential amino acid motif and substrate activation mechanism.[99, 121–123] Furthermore, several recently solved structures of bifunctional plant diterpene synthases (diTPSs) have shown to contain the typical domains of both classes. However, many of these plant diTPSs are classified as monofunctional, as only one of the cyclization activities has been proven to be functional.[99, 121–124] Similar to sesquiterpene cyclization mechanisms, class I diTPSs with two conserved motifs (DDXXD and NSE) catalyze an ionization-dependent cyclization by removal of the pyrophosphate group.[104, 107, 125] In contrast, class II diTPSs typically contain only one characteristic aspartate rich DXDD motif, where the central aspartate represents the catalytic acid for isoprene protonation. The largely hydrophobic active site cavity is composed mainly of aliphatic and aromatic residues, which fold the linear substrate for the upcoming cyclization cascade. Addition of a proton to either a substrate carbon–carbon double bond or an epoxide initiates the reaction cascade. This step yields a tertiary carbocation retaining the pyrophosphate group. Subsequent reactions with other substrate carbon–carbon double bonds form multicyclic structures.[73, 88, 126–128] Depending on the involved carbon atoms and the reaction sequence, various different cyclizations are possible before a proton elimination or water quenching terminates the reaction. Similar to the cyclization of FPP, the basic diterpene structure can be further modified by the action of CYPs and other enzymes.[73, 129]

1.4. INDUSTRIAL SUPPLY OF TERPENES

Commercial large-scale production of isoprenoids or their respective precursors with high economic value is rare and usually relies on the extensive extraction and purification from natural host organism material.[130] A prominent example is astaxanthin, a carotenoid of the tetraterpenes, and responsible for the red color of salmon and crustaceans. Industrial supply as aquaculture feed supplement is currently ensured by cultivation of different natural astaxanthin producing microbes, which have not been genetically engineered, including the alga *Haematococcus pluvialis*.[131, 132] Despite high commercial values, supply of many natural compounds is insufficient because of limited availability.[52–54] Complex mixtures with quite similar physical and chemical properties complicate purification procedures and affect the economic production efficiency.[55, 56] Concurrently, structural complexity, including unsaturated bonds and chiral regions, often leads to a difficult, costly and inefficient chemical synthesis.[133–135]

The rapidly advancing synthetic biology has led to economically attractive ways and alternatives for the future biosynthesis of different high-value chemicals, or at least their structural backbones. The powerful and comprehensive combination of multiple scientific disciplines, including biochemistry, molecular biology, biotechnology, computational biology and bioengineering offers new opportunities for understanding and development of theses sustainable and efficient production pathways. To obtain high production titers and yields of important building blocks, there is an ongoing scientific as well as industrial effort for fast-growing and tractable biological microbial factories.[56, 136] Application of advanced metabolic engineering strategies in industrial workhorse strains such as *E. coli* and *S. cerevisiae*, for which the fermentation strategies are well-established, promise more efficient and cost-effective production routes.[137] Although the biosynthesis of terpenes by evolutionary optimized synthases offers great advantages over chemical synthesis,[138] the currently available product spectrum is limited and covers only a small fraction of possible structures.[139] Plants and especially fungi represent a highly valuable genetic source for future genome mining and discovery of new biotechnological tools.[99, 130] In contrast to plants and fungi as initial production hosts, engineered microorganisms are more accessible for large-scale engineering projects where various different modifications and process parameters may be compared and combined. Flexibility in the biosynthesis of different natural products via the introduction of new genetic sequences or the mutation of existing pathway genes is a great advantage of microbial systems. The development of a stable and

controllable production platform based on microbial fermentation is also attractive, as it has the potential to reduce supply volatility as well as production costs. However, it should not be ignored, that microbes have some technical disadvantages compared to plant systems. In particular the inefficient expression of heterologous P450 systems.[140] In this regard, there is still a major demand for research and development.

Over the last years, there has been remarkable success in engineering the MEP pathway to increase the supply of isoprenoid precursors in *E. coli*, as a widely used platform for terpene production.[67, 130, 141–143] Balancing the cellular pool of accessible G3P and pyruvate or manipulating the expression of key enzymes in the respective pathways resulted in significantly increased terpenoid titers. For instance, the 1-deoxy-D-xylulose 5-phosphate synthase (DXS) or the isopentenyl-diphosphate delta isomerase (idi) represent rate limiting enzymes within the MEP pathway and basic targets for terpene related metabolic engineering.[144, 145] Since the universal precursors DMAPP and IPP are decisive to all isoprenoids, strains with enhanced precursors flux can serve as platform hosts for the production of any natural scaffold, for which the required TPS is characterized and genetically accessible.

So far, the number of candidates for biotechnologically produced terpenoids with sufficient commercial supply is still moderate. Limiting factors are structural complexity or challenging biosynthetic pathways as well as the lack of characterized enzymes. While efforts to obtain high terpene titers were successful for some industrially important compounds such as limonene (2.7 g/L)[146, 147] or α-bisabolol (9.1 g/L),[148] most engineering efforts fail in reaching economic relevant yields. The currently highest published titer of an heterologous produced terpenoid was achieved in a project about the semi-synthesis of the potent malaria drug artemisinin.[149, 150] The corresponding sesquiterpene precursor amorphadiene, which is a key intermediate in the synthesis of artemisinin, was produced with titers of up to 40 g/L in optimized *S. cerevisiae*. A subsequent oxidized intermediate, artemisic acid as a high-grade pharmaceutical precursor, was produced with 25 g/L.[151, 152] Many different approaches have been investigated to achieve the goal of an economic and robust source of high-quality products with minimized environmental impact. Especially adjusted fermentation processes, optimized culture conditions, new extraction methods and a general scale-up usually lead to further increased productivity. A progressing improvement of microbial isoprenoid production therefore requires diverse comprehensive approaches in order to identify restrictive factors and push the entire process to new performance limits.[58, 140]

2 MATERIAL AND BASIC METHODOLOGY

All procedures and materials described in the following section represent a summary and an overview of the most important methods used in this thesis. Basic process steps, media compositions and reagent specifications, which have significantly contributed to the generation of the published data are listed. Further details, process deviations and more specific information on individual aspects and special experimental demands are provided within the individual publication and the respective supplemental data sheets.[66, 115, 153]

2.1. General experimental procedures

2.1.1. Chemicals and reagents

The chemicals used within the included projects were obtained from standard sources at the highest purity grade available. Components for cultivation media were purchased from Roth chemicals and Applichem GmbH. Acetonitrile (ACN), ethyl acetate (EtOAc), ethanol (EtOH) and n-hexane were obtained from Roth chemicals. Enzymes for hydrolysis were provided by Novozymes. Enzymes for polymerase chain reactions (PCR), restriction digestion and ligation were purchased from Thermo Fisher Scientific. DNA preparations kits (Thermo Fisher Scientific) and gel extractions kits (Analytik Jena) were used for DNA processing. Terpene standards for quantification were obtained from Biomol.

2.1.1.1. Wheat bran hydrolysation[66]

Production of 10% (w/v) biomass hydrolysate stock solution required 100 g of dry wheat bran mixed with 500 mL sodium acetate (NaOAc) buffer (50 mM; pH 5). After incubation with 100 µL of amylase enzyme solution (22 °C; 1 h), the mixture was autoclaved (121 °C; 15 min). An enzyme mixture containing 1.0% (v/v) of Cellic® HTec and Cellic® CTec in NaOAc buffer (final volume: 500 mL) was centrifuged for sediment separation and sterile filtrated. The enzyme solution was merged with the wheat bran suspension under sterile conditions for hydrolysis initiation (50 °C; 72 h; moderate shaking). Solid residues were removed by sedimentation and the liquid fraction was sterile filtrated.

For the generation of larger volumes, 5 kg of dry wheat bran and 35 liters of NaOAc buffer were mixed. Initial starch hydrolysis (5 mL of amylase; 37 °C; 1 h) was followed by a sterilization step (121 °C; 30 min; constant stirring). The hydrolysis was carried out with a 3.0% (v/v) enzyme solution of Cellic® HTec and Cellic® CTec in 1.5 L NaOAc

buffer (50 ℃; 48 h; pH of 5; constant stirring). A plate separator (9000 rpm) was applied for solid residue separation, followed by sterile filtration of the clarified hydrolysate.

2.1.1.2. Media composition

M9 MINERAL MEDIUM

H_2O		821.00 mL/L	
Glucose (20%)		40.00 mL/L	0.80% (w/w)
$CaCl_2$ (1 M)		0.20 mL/L	0.20 mM
$MgSO_4$ (1 M)		0.75 mL/L	0.75 mM
M9 salt solution (10x)		100.00 mL/L	
	Na_2HPO_4		33.70 mM
	KH_2PO_4		22.00 mM
	NaCl		8.55 mM
	NH_4Cl		9.35 mM
$FeSo_4$ (5 mM)		25.00 µl/L	0.13 µM
Casamino acids (200 g/L)		25.00 mL/L	0.50% (w/w)
Trace elements solution (100x)		10.00 mL/L	1.00x
Biotin (1 mg/mL)		1.00 mL/L	1.00 µg/L
Thiamin (1 mg/mL)		1.00 mL/L	1.00 µg/L
Antibiotic (1000x)		1.00 mL/L	1.00x

MODIFIED R-MEDIUM[154]

H_2O	up to 1 L	
KH_2PO_4	13.30 g/L	98.00 mM
$(NH_4)_2HPO_4$	4.00 g/L	30.00 mM
Citric Acid	1.70 g/L	8.80 mM
Yeast extract	5.00 g/L	0.50% (w/w)
Glycerol	35.00 g/L	3.50% (w/w)
$MgSO_4$ (1 M)	4.90 mL/L	4.90 mM
Fe(III) citrate (0.1 M)	2.45 mL/L	0.25 mM
Trace elements solution (100x)	10.00 mL/L	1.00x
Thiamin (1 mg/mL)	1.00 mL/L	3.30 µM
Antibiotic (1000x)	1.00 mL/L	1.00x

Optimized hydrolysate medium

H_2O		698.50 mL/L	
$CaCl_2$ (1 M)		0.10 mL/L	0.10 mM
$MgSO_4$ (1 M)		0.25 mL/L	0.25 mM
M9 salt solution (10x)		50.00 mL/L	
	Na_2HPO_4		16.85 mM
	KH_2PO_4		11.00 mM
	NaCl		4.28 mM
	NH_4Cl		4.68 mM
Hydrolysate (10%)		250.00 mL/L	25.00% (v/v)
Antibiotic (1000x)		1.00 mL/L	1.00x

LB medium

H_2O	up to 1 L	
Yeast extract	5.00 g	0.50% (w/w)
Trypton	10.00 g	1.00% (w/w)
NaCl	10.00 g	170.00 mM

R-medium feeding solution

H_2O	up to 1 L	
Glycerol	600.00 g/L	60.00% (w/w)
Collagen hydrolysate	60.00 g/L	6.00% (w/w)
Yeast extract	6.50 g/L	0.65% (w/w)
$MgSO_4$ (1 M)	50.00 mL/L	50.00 mM
Trace elements solution (100x)	15.00 mL/L	1.50x
0.1 M Fe(III) citrate	5.00 mL/L	0.50 mM

M9 SALT SOLUTION (10X)

H_2O	up to 1 L	
Na_2HPO_4	60.00 g/L	420.00 µM
KH_2PO_4	30.00 g/L	220.00 µM
NaCl	5.00 g/L	68.00 µM
NH_4Cl	10.00 g/L	190.00 µM

TRACE ELEMENTS SOLUTION (100X)

H_2O	up to 1 L	
EDTA	5.00 g /L	13.40 mM
$FeCl_3$-$6H_2O$	0.83 g/L	3.10 mM
$ZnCl_2$	84.00 mg/L	0.62 mM
$CuCl_2$-$2H_2O$	13.00 mg/L	76.00 µM
$CoCl_2$-$2H_2O$	10.00 mg/L	42.00 µM
H_3BO_3	10.00 mg/L	162.00 µM
$MnCl_2$-$4H_2O$	1.60 mg/L	8.10 µM

2.1.2. Gene cloning, plasmid construction and culture conditions

All genes were codon-optimized for improved efficiency and expression in *E. coli*. Custom synthesis of PCR primers and genes was performed by Eurofins Genomics. *E. coli* DH5α cultures were grown at 37 ℃ in lysogeny broth (LB) supplemented with the appropriate antibiotic (ampicillin: 100 µg/mL; kanamycin: 50 µg/mL; chloramphenicol: 34 µg/mL).

Standard protocols were used for PCR, enzymatic digestion and ligation. PCR products were purified by 1% (v/m) agarose in TAE buffer gels. If required, appropriate gene bands were isolated and extracted using the innuPREP DOUBLEpure Kit (Analytik Jena) according to manufacturer's protocol. Transformation in chemical competent cells was performed using a heat shock procedure based on standard protocols. The GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used for plasmid isolation and purification according to manufacturer's protocol. All clones were validated by individual test restriction or DNA sequencing (Eurofins Genomics).

2.1.2.1. Sequence-based identification of new terpene cyclases[115]

The selection of new target sequences for the experimental identification of their potential terpene profile was based on sequence-specific motifs:[115, 155–158]

- Highly conserved (N/D)Dxx(S/T)xxxE NSE-triad
- Aspartate-rich D(D/E)xxD motif
- Arginine residue 46 AA upstream of the NSE motif
- RY-dimer ~80 AA downstream of the NSE motif

2.1.2.2. Gene and protein sequences

The following sequences were used in the presented studies:

SESQUITERPENE SYNTHASES:

Copu1 from *Coniophora puteana* (XP_007772164.1)

Copu2 from *Coniophora puteana* (XP_007771895.1)

Copu3 from *Coniophora puteana* (XP_007765978.1)

DITERPENE SYNTHASES:

Cembratriene-ol synthase from *Nicotiana sylvestris*
(CBTS; GenBank: AAS46038.1)

Taxadiene synthase from *Taxus brevifolia* (txs; GenBank: AAK83566.1)

OTHER SEQUENCES:

1-deoxy-D-xylulose-5-phosphate synthase from *E. coli*
(dxs; GenBank: WP_099145004.1)

Isopentenyl pyrophosphate delta isomerase from *Haematococcus lacustris*
(idi; GenBank: AAC32208.1)

Geranylgeranyl pyrophosphate synthetase from *Pantoea ananatis* (crtE;
GenBank: ADD79325.1) or from *Pantoea agglomerans*
(crtE; GenBank: KPA04564.1)

Phytoene desaturase from *Pantoea ananatis* (CrtB; GenBank: AHG94990.1)

Phytoene synthase from *Pantoea ananatis* (CrtL; GenBank: AHG94989.1)

2.2. TERPENE PRODUCTION

2.2.1. Shake flask fermentation (analytical scale)

For the screening of TPS functionality and the production of analytical terpene samples, 35 mL cultures supplemented with the required antibiotic (100 µg/mL ampicillin; 50 µg/mL kanamycin; 34 µg/mL chloramphenicol) were prepared in baffled shake flasks. Depending on the expected product characteristics, either LB medium or M9 mineral medium was applied. M9 mineral medium is preferred for the production of sesquiterpenes in order to avoid interference or superposition with indole during the subsequent GC-MS analysis. Indole exhibits similar retention properties as certain sesquiterpenes and is generated by *E. coli* due to tryptophanase mediated hydrolysation of tryptophan.[159] An overnight culture of pre-engineered *E. coli* harboring the appropriate plasmid was used for inoculation. Depending on the terpene product, cultivations were carried out at 30 °C and 90 rpm shaking for 24 - 48 h (sesquiterpenes) or at 22 °C and 130 rpm shaking for 48 - 72 h (diterpenes) respectively. If necessary, protein expression was chemically induced at an OD_{600} of ~0.6 by addition of 150 µM isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2.2. Bioreactor fermentation (technical scale)[115]

Fermentations for the generation of higher quantities of terpenes were executed in a DASGIP® 1.3 L parallel reactor system (Eppendorf AG) utilizing modified R-medium[154] at 30 °C cultivation temperature. Inoculation was realized with overnight cultures (target OD_{600} of ~0.1). DO was maintained at 30% and regulated by adjusting stirrer velocity (200 - 1000 rpm), oxygen content (up to 100%) and air flow (starting from 0.5 vvm). A constant pH of 7.00 was controlled with 25% aqueous ammonia. Value shift exceeding pH 7.05 resulted in an automatic addition of feed solution (40 mL). OD_{600} and terpene content were measured by periodic sampling.

2.2.3. 50 L fermentation and parallel product depletion (semi-industrial scale)[66]

Large-scale production of CBT-ol was realized by using a 75 L bioreactor (Bioengineering). Two liters of an overnight culture (37 °C; 8 h) were used for inoculation of 32 L optimized hydrolysate medium (target OD_{600} of ~0.1). After reaching an OD_{600} of approximately 12, the cultivation temperature was reduced from 37 °C to 22 °C, followed by protein expression induction with 1 mM IPTG. A constant pH of 7.0

was controlled by 25% ammonium hydroxide and 25% phosphoric acid. DO was maintained at 40% air saturation by adjusting the stirring velocity (300 – 530 rpm) at a constant airflow of compressed air (10 SLM). A feed protocol regulated the continuous addition of pure hydrolysate solution (~40 g/L glucose; 1.5 mL/min; 40 h; subsequent 3 mL/min; until end of process). Cell density and product content were measured regularly.

2.3. TERPENE ISOLATION

2.1.3. Terpene isolation (analytical scale)

For analytical terpene isolation, 35 mL of the respective culture was mixed with 15 mL extraction solution (equal volumes of EtOAc, EtOH and n-hexane). The mixture was shaken for 30 min in a glass flask, followed by a short centrifugation step (1 min; 8000 g). A sample of the clear organic phase was directly analyzed via GC-MS.

2.3.1. Terpene isolation (technical scale)

For terpene extraction from a larger cultivation scale, the culture broth was mixed with an equivalent volume of an EtOAc and EtOH solution (1:1). A thorough mixing step (12 h; 60 rpm) at 22 °C (sesquiterpenes) or 30 °C (diterpenes) within a glass flask, was followed by centrifugation (7000 g; 15 min). After discarding the pellet and adding pure n-hexane (25% of the total volume), the solution was mixed (2h; 60 rpm; 22 °C). A separation funnel was used for phase separation. The upper organic fraction was isolated and concentrated utilizing a rotary evaporator. The residual crude oil was resolved in an appropriate solvent solution depending on the further analytics or purification process steps.

2.3.2. Terpene Isolation (semi-industrial scale)[66]

For a semi-continuous product isolation during a 50 L fermentation, a tailor-made bypass system was utilized. For this purpose one kg Amberlite® XAD®-2 beads (Sigma-Aldrich) was filled into a chromatography column (2 L; 10 × 24 cm), sterilized with NaOH (1 M; 3 h) and neutralized with sterile water. The filled column was connected to the reactor via pump modules, providing constant fermentation broth circulation (120 mL/min). The adsorbent material was flushed (8 L sterile water) and all bound

eluted (1.5 L EtOAc) at regular intervals of 24 h. After every elution step, the column was washed (10 L sterile water) followed by sterile air. All EtOAc volumes were pooled and concentrated utilizing a rotary evaporator.

2.4. PRODUCT PURIFICATION

2.4.1. Centrifugal partition chromatography (CPC) purification[66]

CPC-based terpene purification was performed with a CPC 250 (Gilson Purification), two connected columns and a total column volume of 182 mL at room temperature. Two preparative HPLC pumps (maximal flow rate: 50 mL/min) were used for liquid handling. Product elution was monitored at 210 nm wavelength. Different solvent systems were used for the purification of diterpenes:

CBT-ol: ACN / EtOH / n-hexane: 27.40 / 1.60 / 71.00 (v/v/v)

Taxadiene: ACN / EtOH / n-hexane: 1.07 / 1.00 / 4.33 (v/v/v)

Biphasic systems were prepared by mixing the respective solvent volumes, followed by equilibration (22 °C; 2 h; vigorously shaking). The CPC column was filled with the upper (stationary) phase. The lower fraction served as mobile phase. CPC separation was initiated by an injection of 2 mL crude sample (1700 rpm rotational speed; 8 mL/min mobile phase flow rate) in descending mode. The terpene-containing fractions were pooled and evaporated.

2.4.2. High performance liquid chromatography (HPLC) purification

Sesquiterpene purification and terpene-isomer separation was performed by semi-preparative high-performance liquid chromatography (HPLC: UltiMate™ HPG-3200BX pump, UltiMate™ 3000 AFC and DAD from Thermo Fischer). All chromatograms were recorded at 210 nm wavelength. A NUCLEODUR® C18 HTec 250/10 mm 5 µm column (MACHEREY-NAGEL GmbH & Co. KG) was used for sesquiterpene purification (2.2 mL/min flow rate). 50 mg of crude extract dissolved in two mL of ACN and H_2O (9:1) was used for injection. An initial gradient (90% to 100% ACN within 10 min; 30 °C) was applied, followed by maintaining the 100% ACN level for 30 min. In this experimental setup, β-copaene was separated from the crude extract after 28-29 min as a distinct product peak. Cubebol eluted already after 13-15 min without any prominent peak due to its structural properties. UV adsorption is related to changes in energy levels

of the electrons within the π-bonds and is common for conjugated double bonds (chromophores).[160] δ-Cadinene was isolated within the same setup and eluted after approximately 27 min.

The separation of diterpene-isomers was performed using a Lux® 5 µm amylose-1 LC column (250 × 10 mm; Phenomenex) in an isocratic mode (2.2 mL/min; 50 °C; 15 min). The mobile phase was composed of ACN and H_2O (7:3). Injection volume was 2 mL of CPC pre-purified racemic CBT-ol oil (20 mg/mL) dissolved in ACN and H_2O (7:3).

2.5. ANALYTICS

2.5.1. Gas chromatography-mass spectrometry (GC-MS) & FID analysis

Breakdown and analysis of extracted terpene products was conducted by gas chromatography (GC), utilizing a Trace GC Ultra with DSQII (Thermo Scientific) and a SGE BPX5 column (30 m, I.D. 0.25 mm, film 0.25 µm). Column temperature of 50 °C was kept for 2.5 min, followed by a gradient to 320 °C (10 °C/min). Final temperature was maintained for three min. MS data were recorded at 70 eV (EI) and m/z (rel. intensity in %) as total ion current. Datasets were collected in full scan mode (m/z 50 - 650). Compounds were identified by comparison of product retention times and mass-spectrometric (MS) fragmentation patterns to commercially available standards and databases. For quantification of terpene concentrations, corresponding flame ionization detector (FID) peak areas of each sample were correlated to a defined sesqui- or diterpene standard of known quantity.

2.5.2. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra of the terpene products were recorded in $CDCl_3$ (diterpenes) or C_6D_6 (preferred for some sesquiterpenes) with a Bruker Ascend™ 400 MHz NMR spectrometer. All chemical shifts are relative to $CDCl_3$ at δ = 7.26 ppm ($_1$H-NMR) and $CDCl_3$ at δ = 77.16 ppm (^{13}C-NMR) or C_6D_6 at δ = 7.16 ppm (^{1}H-NMR) and C_6D_6 at δ = 128.06 ppm (^{13}C-NMR).

CHAPTER II
RESEARCH

3 Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents

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By Wolfgang Mischko, Max Hirte, Simon Roehrer, Hannes Engelhardt, Norbert Mehlmer, Mirjana Minceva and Thomas Brück

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3.1. AUTHORS' CONTRIBUTIONS

WOLFGANG MISCHKO: General coordination of the research collaboration (Institute of Bioprocess Engineering, Biothermodynamics and the Werner Siemens-Chair of Synthetic Biotechnology). Planning and execution of experiments, in particular the utilization and characterization of the CBT-ol TPS as well as the evaluation and optimization of a suitable *E. coli* based terpene production system with enhanced product yield. Furthermore, the development of an efficient enzyme system for the hydrolytic utilization of wheat bran as a sustainable nutrient medium for *E. coli* and the biotechnological CBT-ol production in a semi-industrial reactor production scale. Moreover, the development and evaluation of new sustainable terpene purification routes including parallel adsorption, HPLC and CPC with special regard to the screening and identification of suitable solvent systems for CBT-ol separation. Analysis and evaluation of terpenoids as well as conduction of *in vivo* bioassays to examine the biological activity and target range of CBT-ol. Finally, comprehensive data analysis, supervision of students and preparing the entire manuscript.

MAX HIRTE: Comprehensive support and consulting during the entire project, in particular in the development of suitable solvent systems for diterpene separation as well as establishing methods, processes and analytics for heterologous terpene production and characterization.

SIMON ROEHRER: Execution and maintenance of the CPC-based purification system. Cooperation in screening and developing suitable biphasic solvent systems for CBT-ol separation.

HANNES ENGELHARDT: Execution of cell-based bioassays and evaluation of biological activities.

NORBERT MEHLMER: Coordination, support and supervision of the research project.

MIRJANA MINCEVA: Supervision of the CPC-based product purification as well as critical revision of the manuscript.

THOMAS BRÜCK: Drafting of the research objectives, project lead and cooperation in writing the manuscript as well as its critical revision.

3.2. SUMMARY

Modern agriculture that aims to sustainably supply a constantly growing world population also requires sustainable solutions to emerging challenges. In this context, there is an increasing demand for eco-friendly alternatives to conventional chemical agrochemicals and feedstocks. The study "Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents" describes the development and optimization of a sustainable process for the biotechnological production and purification of the complex diterpene CBT-ol. This natural compound represents an essential part of the tobacco (*Nicotiana sp.*) plant's defense against insects and is therefore of importance as a biological insect repellent. Favorable biodegradability and target selectivity are product specific characteristics and make CBT-ol a sustainable alternative to conventional chemical insecticides.

The entire production process relies on the utilization of a complex and cost-efficient wheat bran-based fermentation medium. This was realized by the development of a suitable enzyme system for optimized biomass hydrolysis and conversion. Heterologous gene expression and metabolic engineering, including RBS combinatorics for an optimized polycistronic operon led to efficient terpene production in *E. coli*. Utilizing the waste stream-based growth medium, the metabolically balanced microbial system is capable of generating 78.9 ± 2.4 mg/L CBT-ol in a 50 L bioreactor, which exceeds all previously reported production titers. In addition, a new ecologically advantageous downstream processing strategy was established, combining an adsorption-based extraction method, centrifugal partition chromatography (CPC) and HPLC-based isomer separation. This process, as a whole, enables the *in situ* extraction of the target molecule during the fermentation as well as 95% CBT-ol recovery and purification in a single CPC step. As a result, solvent consumption is significantly reduced in comparison to conventional methods. This is the first demonstration for CPC based purification of *E. coli* derived terpenoids. The subsequent application of chiral HPLC chromatography allowed for the isolation of the two isomers α- and β-CBT-ol. Various *in vivo* and *in vitro* bioactivity studies including yeast, bacteria and human cancer cell lines verified the insecticidal effect of CBT-ol but also indicated biological activity specifically targeting gram-positive bacteria. These results allow a potential utilization in even more industrially relevant areas.

3.3. Press Review

Last checked: 10.07.2018

1. Bio-Insektenspray für die Landwirtschaft

„Biologisch abbaubare Pflanzenschutzmittel ohne Risiken und Nebenwirkungen“

2018-06-06 Technische Universität München - Forschung

Available at: https://www.tum.de/die-tum/aktuelles/pressemitteilungen/detail/article/34679/

2. Organic insect deterrent for agriculture

“Biodegradable crop protection products without risks or side effects”

2018-06-06 Technical University of Munich - Research news

Available at: https://www.tum.de/nc/en/about-tum/news/press-releases/detail/article/34679/

2018-06-06 Technical University of Munich - Department of Chemistry

Available at:
http://www.chemie.tu-muenchen.de/en/research/highlights/organic-insect-deterrent-for-agriculture/

2018-06-06 SeedQuest

Available at:
https://www.seedquest.com/news.php?type=news&id_article=98682&id_region=&id_category=202&id_crop=

3. Neues Bioinsektizid vergrämt Schädlinge

„Einen fundamentalen Wechsel im Pflanzenschutz glaubt Professor Thomas Brück von der TU München mit einem Bio-Insektenspray einläuten zu können.“

2018-06-06 top agrar Österreich

Available at: https://www.topagrar.at/ackerbau/Neues-Bioinsektizid-vergraemt-Schaedlinge-9211479.html

4. Bio-Insektenspray für die Landwirtschaft

2018-06-06 Technische Universität München - Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Available at:
http://www.weihenstephan.de/index.php?id=9&tx_ttnews%5Bpointer%5D=1&cHash=e123899a4a3100dec92525
1146378035

5. Biologisches Spray gegen Blattläuse

2018-06-06 Argrarzeitung

Available at: https://www.agrarzeitung.de/nachrichten/politik/forschungsprojekt-biologisches-spray-gegen-blattlaeuse-82817?crefresh=1

6. Biodegradable crop protection products without risks or side effects

2018-06-06 idw - Informationsdienst Wissenschaft

Available at: https://idw-online.de/de/news696993

2018-06-06 Innovations report

Available at: https://www.innovations-report.com/html/reports/life-sciences/biodegradable-crop-protection-products-without-risks-or-side-effects.html

2018-06-07 PHYS.org

Available at: https://phys.org/news/2018-06-biodegradable-crop-products-side-effects.html

7. A tobacco-derived insect repellent – for crops

2018-06-07 New Atlas

Available at: https://newatlas.com/tobacco-insect-repellent-crops/54962/

8. Synthetic Biotechnology Tools Used to Create a Biodegradable Insecticide

2018-06-07 Engineering360

Available at: https://insights.globalspec.com/article/9030/synthetic-biotechnology-tools-used-to-create-a-biodegradable-insecticide

9. Biologisch abbaubare Pflanzenschutzmittel ohne Risiken und Nebenwirkungen

2018-06-07 Netzwerk-forum zur Biodiversitätsforschung Deutschland

Available at: http://biodiversity.de/en/news/bio-div/biologisch-abbaubare-pflanzenschutzmittel-ohne-risiken-nebenwirkungen

2018-06-07 Topagrar

Available at: https://www.topagrar.com/news/Acker-Agrarwetter-Ackernews-Biologisch-abbaubare-Pflanzenschutzmittel-ohne-Risiken-und-Nebenwirkungen-9210004.html

10. Bio-Spray vergrault Blattläuse

„Forscher entwickeln unbedenkliche Alternative zu konventionellen Insektiziden“

2018-06-07 scinexx

Available at: http://www.scinexx.de/wissen-aktuell-bild-22815-2018-06-07-36530.html

11. Biotech-Wirkstoff vergrämt Läuse

2018-06-07 Transkript

Available at: https://transkript.de/news/biotech-wirkstoff-vergraemt-laeuse.html

12. A tobacco-derived insecticide – for crops

2018-06-07 vaaju

Available at: https://vaaju.com/aus/a-tobacco-derived-insecticide-for-crops/

13. Ecologically Harmless Insect Repellent

2018-06-07 Laborpraxis Worldwide

Available at: https://www.lab-worldwide.com/ecologically-harmless-insect-repellent-a-722524/

14. Germans discover insect repellent

“German researchers have extracted an insect repellent from tobacco plants that might scare away pests, such as aphids”.

2018-06-07 Ontario Farmer

Available at: http://www.ontariofarmer.com/sitepages/?aid=12990&cn=Quicklinks&an=Germans%20discover%20insect%20repellent

15. A tobacco-derived insect repellent - for vegetation

2018-06-08 Frontal Coverage

Available at: http://frontalcoverage.com/news/775829/A-tobacco-derived-insect-repellent---for-vegetation

2018-06-08 Political Wired

Available at: http://politicalwired.com/news/775829/A-tobacco-derived-insect-repellent---for-vegetation

16. A tobacco-derived insect repellent – for crops

2018-06-09 Weird news

Available at: http://weirdnews.info/2018/06/09/a-tobacco-derived-insect-repellent-for-crops/

17. TOBACCO PLANT INSPIRES RESEARCHERS TO DEVELOP BIODEGRADABLE CROP PROTECTION

2018-06-10 Biofuels Digest

Available at: http://www.biofuelsdigest.com/bdigest/2018/06/10/tobacco-plant-inspires-researchers-to-develop-biodegradable-crop-protection/

18. ‚MÜCKENSPRAY' GEGEN SCHÄDLINGE

2018-06-11 Innovation Origins

Available at: https://innovationorigins.com/de/mueckenspray-gegen-schaedlinge/

19. NON-TOXIC TOBACCO INSECT REPELLENT

"An innovative insect repellant derived from tobacco could offer an eco-friendly alternative to toxic pesticides."

2018-06-11 ideaCONNECTION

Available at: https://www.ideaconnection.com/new-inventions/non-toxic-tobacco-insect-repellent-13166.html

20. BIO-INSEKTENSPRAY GEGEN SCHÄDLINGE

„TUM entwickelt biologisch abbaubares Pflanzenschutzmittel"

2018-06-12 Gärtner-Florist | Aktuelles

Available at: http://www.gaertner-und-florist.at/?id=2500%2C5566618%2C%2C

21. ORGANIC INSECT DETERRENT FOR AGRICULTURE

2018-06-12 Seed Daily Farm news

Available at: http://www.seeddaily.com/reports/Organic_insect_deterrent_for_agriculture_999.html

22. INSEKTENSPRAY FÜR PFLANZEN

„Ein Forschungsteam entwickelt ein biologisches Abwehrspray gegen Schädlinge ohne Böden und Gewässer zu verunreinigen."

2018-06-13 Vollwerther

Available at: https://www.voll-werther.de/news/insektenspray-fuer-pflanzen/

2018-06-13 energie|tipp

Available at: https://www.energie-tipp.de/news/insektenspray-fuer-pflanzen/

23. Biologische Pflanzenschutzmittel gegen Blattläuse

2018-06-13 Bayrischer Rundfunk BR

Available at: https://www.br.de/themen/wissen/insektenabwehr-pflanzenschutzmittel-biologisch-cembratrienol-schaedlinge-100.html

24. Bio-based insect repellent banishes aphids

"Researcher at Munich's TU have developed a bio-based and biodegradable crop protection agent based on CBT-ol, a chemical in the leaf of the tobacco plant."

2018-06-13 Bioökonomie.de

Available at: https://biooekonomie.de/en/nachrichten/bio-based-insect-repellent-banishes-aphids

25. Repelent z tabáku by se mohl stát netoxickým postřikem proti hmyzu (The non-toxic tobacco repellent against insects)

2018-06-14 stoplusjednicka

Available at:
https://www.stoplusjednicka.cz/repelent-z-tabaku-se-mohl-stat-netoxickym-postrikem-proti-hmyzu

26. Repelente de insectos biodegradable y que no causa daños ecológicos (Biodegradable insect repellent that does not cause ecological damage)

2018-06-19 Noticias de la Ciencia y la technologia

Available at: http://noticiasdelaciencia.com/not/29051/repelente-de-insectos-biodegradable-y-que-no-causa-danos-ecologicos/

27. Neues Insektizid schützt das Leben von Bienen

2018-06-20 Trends der Zukunft

Available at: https://www.trendsderzukunft.de/neues-insektizid-schuetzt-das-leben-von-bienen/

28. Duitse wetenschappers ontwikkelen biologisch bestrijdingsmiddel tegen insecten (German scientists develop biological pesticides against insects)

2018-06-21 AGF.nl

Available at: http://www.agf.nl/artikel/175546/Duitse-wetenschappers-ontwikkelen-biologisch-bestrijdingsmiddel-tegen-insecten

29. Ein rundum ökologischer Pflanzenschutz

„Duftstoff der Tabakpflanze hält Blattläuse fern“

2018-06-25 Pflanzenforschung.de

Available at: https://www.pflanzenforschung.de/de/journal/journalbeitrage/ein-rundum-oekologischer-pflanzenschutz-duftstoff-der-t-10941

30. Protecting plants by deterrents instead of killing insects

2018-06-26 TreeHugger

Available at:
https://www.treehugger.com/biomimicry/protecting-plants-deterrents-instead-killing-insects.html

31. Chất chống côn trùng hữu cơ dành cho nông nghiệp (Organic insect repellent for agriculture)

2018-06-29 Vietnam Academy of Agricultural Sciences

Available at: http://vaas.org.vn/chat-chong-con-trung-huu-co-danh-cho-nong-nghiep-a17903.html

32. New spray to keep the insects at bay

“A biodegradable agent that keeps pests at bay without poisoning them is being touted as a lifesaver for organic producers losing the perpetual battle against crop-consuming critters.”

2018-07-01 FarmWeek

Available at: https://farmweek.com/new-spray-to-keep-the-insects-at-bay/

33. Environmentally Friendly Insect Repellent for Agriculture

“Cembratrienol (CBTol) protects plant from insects”

2018-07-09 GLOBAL PLANT PROTECTION NEWS

Available at: https://iapps2010.me/2018/07/09/cembratrienol-cbtol-protects-plant-from-insects/

34. Biodegradable crop protection products

2018-/-/ European Business & Biodiversity Campaign

Available at: https://www.business-biodiversity.eu/36681/Newsdetailseite/ebbc_index01.aspx?newsid=71902&newsrefid=36715&row=0&newsrefaddcoid=&nafrom=&nato=

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Modular biomanufacturing for a sustainable production of terpenoid-based insect deterrents†

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Synthetic agricultural insecticides are toxic to many species and accumulate in the environment. Therefore, the development of target-specific and biodegradable insecticides and deterrents is in demand. This study describes an improved and sustainable process for the green production of a biological insect repellent based on the diterpene cembratriene-ol (CBT-ol). This compound is a natural part of the tobacco (*Nicotiana* sp.) plant's defense against insects and thus minimizes damage to the environment. The study reports a new recombinant (*E. coli*) CBT-ol production and purification system. Efficient production was achieved by ribosomal binding site combinatorics using the BioBrick assembly system. These methods generated a metabolically balanced microbial system capable of generating 78.9 ± 2.4 mg L^{-1} CBT-ol in a 50 L bioreactor. Fermentations were entirely carried out on enzymatically generated wheat bran hydrolysate, representing a waste fraction of the grain milling process. The application of this complex and cost-efficient cultivation medium enabled an ecologically and economically sensible production of this high-value insect deterrent. Moreover, an ecologically favorable downstream processing protocol was established, combining adsorptive CBT-ol capture and centrifugal partition chromatography (CPC) followed by HPLC-based isomer separation. This is the first report using CPC to recover recombinant-generated, bioactive terpenes. The methodology enabled 95% CBT-ol recovery and purification in a single CPC step with significantly reduced solvent consumption in comparison to conventional chromatographic methods. *In vivo* and *in vitro* bioactivity studies confirmed the insecticide characteristics but also indicated that CBT-ol shows other bioactivities specifically targeting Gram-positive bacteria.

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Introduction

With over 50 000 characterized compounds, terpenoids are the largest and structurally most diverse group of natural products. With respect to the development of a sustainable bioeconomy, terpenoids represent important renewable chemical building blocks with industrial applications in the pharmaceutical (*e.g.*, taxol[1]), cosmetic (*e.g.*, pseudopterosin[2]), food (*e.g.*, β-carotene[3]), fragrance (*e.g.*, nootkatone[4]), and chemical (*e.g.*, limonene[5]) industries. Like most bioactives, terpenoids are secondary metabolites, only found in very low abundance in the natural source.[6] As most industrially relevant terpenoids are derived from plant material, their extraction poses significant challenges due to the presence of multiple contaminants. To obtain high titers of specific terpenoids, there is an ongoing effort in the scientific community to generate important building blocks by genetic engineering in microbial hosts, such as *E. coli* and *S. cerevisiae*.[7] While these efforts have been successful for some industrially important compounds such as limonene (2.7 g L^{-1})[8,9] and nootkatone (208 mg L^{-1}),[10] most engineering efforts do not generate industrially relevant titers of the desired target compounds. Moreover, reported studies conventionally do not address issues of process scaling and downstream purification of target molecules under economic constraints. The current study addresses issues of sustainable heterologous production and purification at the laboratory and technical scales for a diterpene-type insect repellent from agricultural waste streams targeted at the agrochemical industry.

In the context of the global insecticide market, chemically synthesized active compounds, such as artificial pyrethrum derivatives and most prominently neonicotinoids (*e.g.*, imidacloprid, thiamethoxan and clothianidin; worth US $1.89 billion[11]), are dominant, fast-acting but non-target discriminant products. Neonicotinoids represent the major class of

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†Electronic supplementary information (ESI) available: Gene and protein sequences, additional figures, experimental details and copies of ^{1}H NMR and ^{13}C NMR spectra. See DOI: 10.1039/c8gc00434j

insecticides that mediate neurotoxic effects in all insects through irreversible binding to nicotinic acetylcholine receptors.[12,13] Therefore, these compounds affect pests as well as beneficial insects such as bees and bumble bees, thereby negatively impacting pollination of agricultural crops and biodiversity in rural regions.[13,14] Chemical insecticides even endanger industrial agricultural activity and the sustainability of a still growing human population. Moreover, these chemicals are poorly biologically degradable,[13,15] which leads to an accumulation in the environment resulting in a negative impact on the biodiversity of terrestrial and aquatic ecosystems.[16] The broad environmental effects of neonicotinoids has recently triggered the European Commission to strictly limit the applicability of these compounds in agricultural activities.[17–19] By contrast, bio-based insecticides are rapidly degraded by terrestrial microbes or light and therefore do not accumulate. In addition, selected natural insecticidal compounds are non-toxic to off-target insects, ensuring only positive effects on agricultural activity and crops yields.

In this context, the class of cembranoid diterpenes, originally reported from cuticular wax of many *Nicotiana* species, has been identified as a promising resource. The two most abundant epimeric tobacco cembranoids are (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol and (1*S*,2*E*,4*R*,6*R*,-7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (CBT-diol) with the typical macrocyclic C_{14} backbone.[20,21] These are constituents of the plant's exudates[22] representing a major part of the plant's defense against insects, pathogenic microbes and herbivores.[23] Additionally, several other bioactivities encompassing anti-tumor,[24] antibiotic[25] or neuroprotective[26] properties have been reported. Interestingly, the mono-hydroxylated biosynthetic precursors of these compounds, cembratriene-ol (CBT-ol), could only be detected in trace amounts *in planta*. However, CBT-ol has recently received some attention[20,27] due to its apparent insecticidal activity but data are scanty.

CBT-ol occurs as α- and β-2,7,11-cembratriene-4-ol isomers (α-CBT-ol, also known as thunbergol or isocembrol and β-CBT-ol, equivalent to 4-epiisocembrol) with absolute configurations determined as (1*S*,2*E*,4*R*,7*E*,11*E*)- and (1*S*,2*E*,4*S*,7*E*,11*E*)-2,7,11-cembratriene-4-ol, respectively.[28,29] The corresponding CBT-ol synthase (CBTS) has been reported from *Nicotiana tabacum* and *Nicotiana sylvestris* with almost identical amino acid sequences. The enzyme is a 58 kDa monomeric[29] class I terpene synthase with the signature aspartate-rich DDXXD and (N,D)DXX(S,T)XXXE motifs, and a magnesium cluster, which is essential for the ionization of an isoprenoid diphosphate group to generate a reactive carbocation intermediate.[30] In nature, the subsequent step in the native pathway is a site-specific oxidation by an also specified cytochrome P450 monooxygenase. The transcriptional silencing of this CYP450 increases the amount of available CBT-ol in the plant exudates and simultaneously enhances the resistance to aphids.[20,31] Other results demonstrate the direct application of purified CBT-ol and its potential as a contact insecticide.[32]

CBT-ol and related cembrene-like compounds derived from the tobacco plant are susceptible to biodegradation and are rapidly catabolized within the maturing plant.[21,33] Additionally, field studies demonstrated the natural degradation process of exogenously applied CBT-ol without an accumulation in the ecosphere.[34] These results contrast those of synthetic neonicotinoids, which are highly toxic and accumulate in soil or water ecosystems.[13,15,16] Therefore, the use of CBT-ol as an ecologically friendly and rapidly biodegradable insecticide represents a radically new, sustainable crop protection strategy, that does not affect beneficial insects populations.

Due to the low concentration *in planta* (0.18% of the leaf dry weight[20]) and the presence of various functionalized cembrene derivatives,[29] the scale-up and purification of the bioactive CBT-ol from plant biomass is technically challenging and associated with high costs. To simplify this process, a more targeted approach is the direct biosynthesis of bioactive cembranoids in an engineered microbial host.[7,35] The recent developments in synthetic biology and bioprocess engineering provide a directed route to obtain tailor-made bioactive compounds, which even improve on cumbersome chemical synthesis routes for structurally complex terpenoids.[36]

The costs and the sustainability of fermentation are still a central obstacle in the economical production of heterologously generated natural products. Utilization of complex biomass hydrolysates derived from agricultural waste streams may provide an alternative toward economically viable production processes. In this respect, wheat bran, a waste stream of the grain processing industry encompassing a production volume of more than 7 million tons per year in the EU[37] may present a formidable resource for the generation of cost-effective fermentation media. Wheat bran predominantly consists of non-starch polysaccharides (up to 45% w/w) but in contrast to other agricultural residues (*e.g.*, straw and wood) it lacks significant amounts of lignin.[37,38] Therefore, wheat bran may be more amenable to enzymatic hydrolysis, which was extensively investigated in this study.

In addition to fermentation media, the recovery of terpenoid target molecules from the fermentation broth is a particular challenge as critical concentrations may affect the production host and the received titers. In this respect, a tailor-made capturing method was evaluated and combined with solid support-free liquid–liquid chromatography, better known as centrifugal partition chromatography (CPC), representing a suitable alternative to conventional separation methods. This flexible technique does not require expensive HPLC column material and provides a high loading capacity in conjunction with efficient sample recovery. The method can be regarded as a more sustainable chromatography method as solvent consumption is significantly reduced compared to the alternatives.[39,40] As this methodology allows for tailor-made biphasic solvent systems, CPC is a rather versatile technology to purify molecules varying in size, polarity or chemical functionality.[39,41] While CPC has been applied in many cases for the purification and separation of bioactive compounds

from plant biomass,[42] this is the first account of the purification of a heterologously produced diterpene in microbial cells.

The total chemical synthesis of the cembrene type macrocyclic core relies on petrochemical educts, which are assembled in at least 8 consecutive reaction steps.[43] This synthesis path involves several intermediate and waste streams. Various toxic, irritant and organ damaging reagents (*e.g.*, oxalyl chloride and triphenylphosphine) and hazardous solvents such as THF, DMF and HMPA are applied. Moreover, the synthesis steps are dependent on the application of several protecting moieties (*e.g.*, TBSCl). As reactions are carried out at temperatures varying between −78 °C to +50 °C, the energy efficiency of the synthesis route is limited.[43,44] An equivalent scenario is reported for the chemical synthesis routes of neonicotinoid type insecticides. The use of hazardous reactants, intermediates and solvents, which are particularly harmful for the environment, is indispensable as well.[45] In contrast, our new biotechnological production process strictly adheres to the 12 principles of green chemistry.[46] Most prominently, we rigorously apply the principles of preventing waste, the use of renewable feedstocks and catalysis as well as the reduction of derivatives.

For the first time, this study presents a holistic production process for a bioactive diterpene insect-deterrent using an engineered *E. coli* production host cultivated on enzymatically generated wheat bran hydrolysate (Fig. 1). In contrast to the chemical multi-step synthesis of the cembrene macrocycle,[43,44] we engineered a consolidated whole cell biocatalyst that enables the single step synthesis in conjunction with a site-specific hydroxylation. Moreover, the biotechnological approaches applied in our study provides a hazardless synthesis route that uses benign chemicals, benign auxiliaries and is designed for energy efficiency. With respect to production host engineering, this study presents a combinatorial ribosome binding site-centered approach for the harmonization of biosynthetic enzyme expression levels. That ultimately allows for an optimized metabolic flux of terpene-relevant intermediates resulting in high target product titers. Additionally, it was demonstrated that the target compound CBT-ol could be purified from crude fermentation broth using a combined adsorptive bypass system in conjunction with a customized CPC methodology. The purified CBT-ol target compound was structurally characterized and respective isomers were tested for their bioactivity in insect deterrent and continuative bioassays. This is the first account of an *E. coli*-based terpene production system cultivated on wheat bran milling waste without accumulating toxic and persisting waste streams. The application of this agricultural residue does not impact agricultural activity *per se* and has no negative influence on food production. We demonstrate that CBT-ol serves as an effective insect deterrent that has no direct toxic effects on the target organisms and may therefore serve in organic farming.

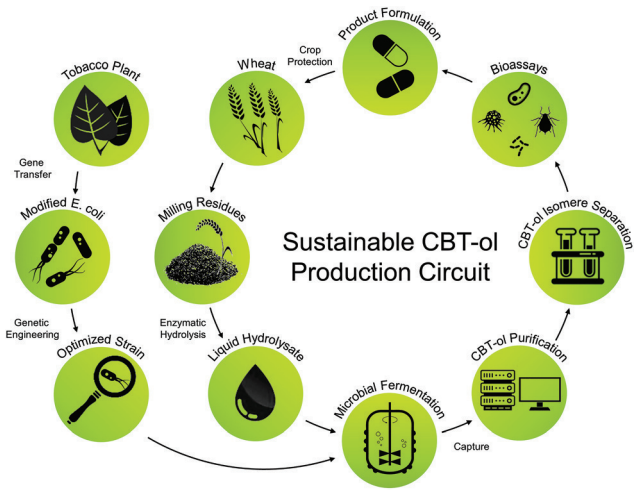

Fig. 1 Graphical presentation of the holistic production process for the sustainable generation of CBT-ol. This process combines the application of an engineered host organism and the circular product generation based on the utilization of the wheat bran waste stream.

Results and discussion

The new agrochemical biorefinery setting was developed according to subsequent unit operations. One main operation was the construction of a suitable high-performance *E. coli* production system by metabolic engineering.

Establishing cembratriene-ol biosynthesis in *E. coli*

In order to develop the heterologous production and processing of CBT-ol, the codon-optimized open reading frame of the previously reported CBTS (lacking the 52 AA plastid transit peptide) from *Nicotiana sylvestris* (AAS46038.1) was co-expressed with an essential geranylgeranyl pyrophosphate synthetase (GGPPS) gene in *E. coli.* A pETDuet-1 vector carrying two strong T7 promotors and integrated ribosomal binding site (RBS) sequences was used for the maximal expression rate of the enzymes resulting in the pETDuet-GGPPS-CBTS vector. The initial gene construct was transformed into *E. coli* HMS 174 (DE3), which was subsequently cultivated (37 °C, 48 h) in artificial M9 minimal medium. Subsequently, the entire fermentation broth with cell mass was extracted with ethyl acetate. In the resulting crude extract, CBT-ol could be identified *via* Gas Chromatography-Mass Spectrometry (GC-MS) analysis by comparison to a commercial standard and to the respective NIST Database entry. The quantification by Gas Chromatography-Flame Ionization Detector (GC-FID) indicated a CBT-ol concentration of 0.92 mg L^{-1}. The action of the CBTS enzyme on the universal diterpene precursor GGPP typically generates two isomers.[20] However, since α- and β-CBT-ol isomers cannot be separated *via* GC-MS, they result in a single product peak (Fig. 3A).

Optimization of cembratriene-ol production by designing a new synthetic polycistronic operon using RBS combinatorics

To increase the CBT-ol production efficiency, the entire production system had to be redesigned on a genetic level. In this regard, it was essential to primarily enhance the supply of the universal terpene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the native *E. coli* non-mevalonate (MEP) isoprenoid pathway. To that end, the major enzymatic bottlenecks 1-deoxy-D-xylulose-5-phosphate synthase (dxs) and IPP isomerase (idi) were overexpressed (Fig. 2A). This measure has previously been reported to increase heterologous terpene yields.[47] All four genes were placed under the control of only one inducible tac promoter due to its benefits in controlling a whole operon without the danger of overcharging the cells like the previous T7 promotor.

To reduce the metabolic burden on the microbial host, we opted to generate a single plasmid containing a polycistronic operon, which allowed for the minimization of the required antibiotics selectors, thereby reducing cellular stress.[48] Moreover, heterologous production *via* non-native metabolic pathways are often limited by the relative biosynthetic enzyme expressions. Therefore, the respective enzyme expression levels must be fine-tuned to balance the system and increase target productivity.[49] In this regard, the order of enzyme operators within an operon structure can be altered.[50] Alternatively, their relative expression rates can be harmonized in a combinatorial approach by varying the strength of the respective ribosomal binding sites (RBS).[49] This leads to different possibilities in the design and optimization of metabolic pathways to ensure high enzyme activities and to avoid protein burden or the accumulation of toxic intermediates. In this study, we opted to

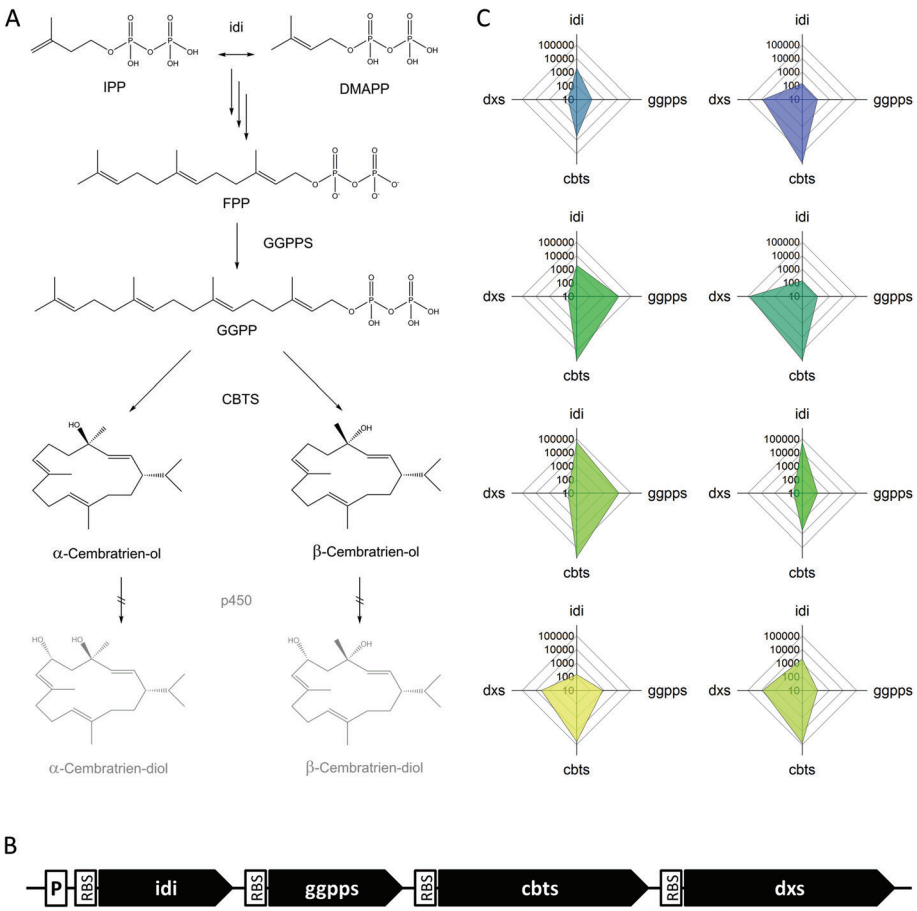

Fig. 2 [A] The metabolic pathway for the production of CBT-ol in *E. coli* consists of the native upstream non-mevalonate (MEP) isoprenoid pathway and a heterologous downstream terpenoid pathway. The structural diversity of all terpenes is derived from two universal isoprenoid C_5 building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to form farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}). GGPP represents the substrate for the cyclization reaction by the CBT-ol synthase (CBTS) providing the two isoforms α- and β-CBT-ol. In the tobacco plant, the further formation of the final α- and β-CBT-diols is catalyzed by a P450 hydroxylase. [B] Design of the synthetic operon for an increased precursor flux and CBT-ol production. Two enzymatic bottlenecks within the upstream MEP pathway (dxs, idi) and the heterologous downstream genes for the GGPP synthase (ggpps) and CBTS were combined in the synthetic operon (idi-ggpps-CBTS-dxs). The pathway is placed under the control of an inducible tac promoter (P). [C] Different exemplary combinations of the operon library illustrated by radar diagrams show RBS-dependent changes in the specific translation rate (au) of all involved genes within the polycistronic operon. The modulation of every enzyme expression level is essential to ensure a balanced pathway function and process efficiency.

leave the order of enzyme operators within the operon structure unaffected, while varying the respective RBS strength for each element in a combinatorial manner. All selected RBS were categorized by their translation initiation rate (au) using the RBS calculator tool[51,52] depending on the associated gene spanning a range from about 50 au up to 500 000 au. Each enzyme operator encoded in the synthetic CBT-ol operon structure was randomly paired with a preselected RBS set (Fig. 2B). We applied the BioBrick cloning strategy designated for the assembly of synthetic operons.[49,53] As a result, a widespread library of different combinations was combinatorially assembled and transformed into *E. coli* HMS 174 (DE3) (Fig. 2C). Subsequently, in excess of 200 clones were screened for CBT-ol production titers. The CBT-ol concentration varied greatly with the changing expression levels within this library. The clone with the highest CBT-ol productivity and constant cell growth was selected for further experiments. Detailed characterization of this clone demonstrated that the optimized translation rate combination for CBT-ol centered biosynthetic genes was: idi (830 au); ggpps (2829 au); CBTS (60 688 au); dxs (167 au). Interestingly, these relatively low translation values indicate that construct stability and production efficiency increase when the cellular burden with heterologous proteins is low. Moreover, the low expression of heterologous proteins also fostered an increased metabolic balance within the cellular system. The new strain carrying the optimized operon (pSB4K5-CBT) was used for further evaluation of the CBT-ol production. Three-day shake flask cultures (50 mL) resulted in one dominant peak in a GC-MS chromatogram (Fig. 3B) with CBT-ol concentrations of up to 13.9 mg L^{-1}. Subsequent, preparative TLC purification resulted in a colorless, highly viscous liquid. This liquid was characterized by nuclear magnetic resonance spectroscopy (NMR) as a mixture of the α- and β-CBT-ol isoforms (Fig. 3D). These isoforms are commonly termed thunbergol and 4-epiisocembrol, respectively.

A comparison of CBT-ol product titers showed that the synthetic CBT-ol operon with harmonized RBS binding sites provided a 15-fold product increase with respect to the primary expression system (0.91 mg L^{-1}). The RBS optimized operon was used for further technical scale-up, purification and bioactivity studies.

Enzymatic wheat bran hydrolysate as alternative nutrition supply

To enable a sustainable production of terpenoids that does not impact land use and agricultural activity, we focused on wheat bran as a feedstock, which constitutes up to 19% of the total grain composition[54] and is considered a major by-product of flour milling. In this study, we had access to this milling residue and devised an enzymatic process for its hydrolysis and use as fermentation medium for *E. coli*. Therefore, the procedure is in line with the concept of generating a sustainable agrochemical from the field for the field. The use of wheat bran hydrolysate as a carbon source has been reported previously in another context.[55] Prior to tailoring the enzyme system for wheat bran hydrolysis, we confirmed the reported biomass composition range of the present wheat bran feedstock: ash 5.2% (3.9–8.10%[56]) (w/w), protein 19.8% (9.60–18%[56]) (w/w) and carbohydrates 65.2% (cellulose 11–13%;[37] hemicellulose 27–31%;[37] starch 18–20%[37]) (w/w). The amounts of lignin and lipids were measured as 5.5% (w/w) and 3.7% (w/w), respectively. Much like other agricultural residues (*e.g.*, straw), wheat bran requires physical pretreatment, a preprocessing step that improves enzyme access to the cellulose.[57,58] In this study, we selected a mild hydrothermal pretreatment (121 °C, 15 min). This methodology did not result in any sugar degradation products and was environmentally and energetically favorable due to the lack of chemical additives (*e.g.*, acid or base).[58] The subsequent enzymatic hydrolysation (combined amylase, cellulase

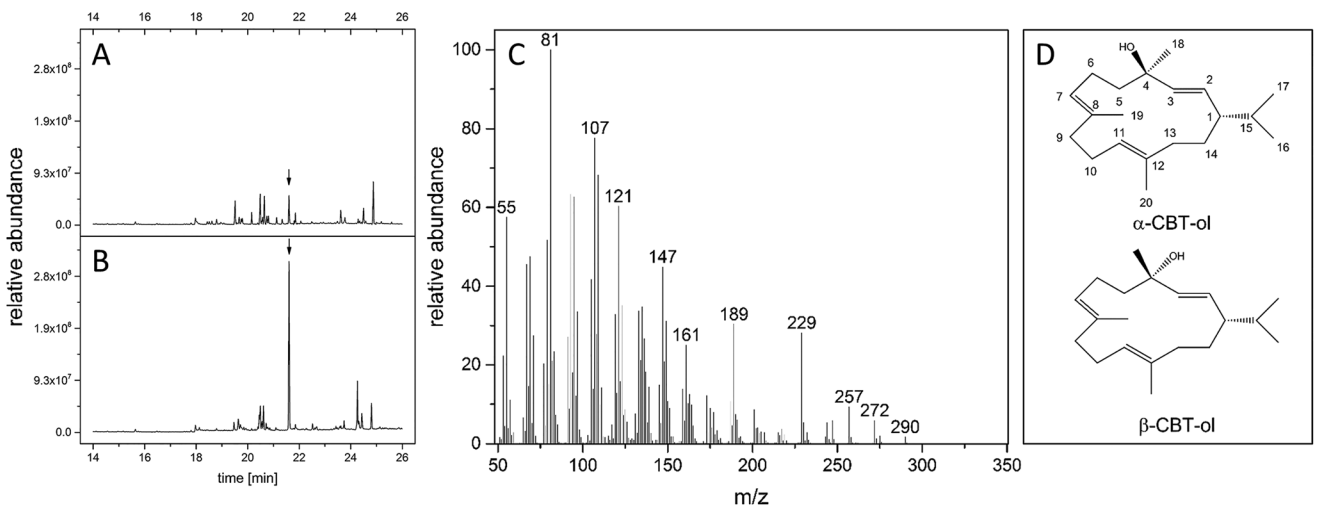

Fig. 3 Gas chromatographic profiles of extracts from *E. coli* cultures transformed with [A] pETDuet-GGPPS-CBTS and [B] the optimized pSB4K5-CBT plasmid showing a dominant product peak at 21.72 min RT. The optimized system presents a significantly higher CBT-ol concentration. [C] The associated mass peaks of the produced CBT-ol, which consists of two isomeric forms with differently oriented hydroxyl groups [D].

and hemicellulase system) of a 100 g L^{-1} wheat bran solution released 33.0 g L^{-1} glucose, 20.5 g L^{-1} xylose and 1.3 g L^{-1} arabinose. The total sugar content of the final wheat bran hydrolysate was 548 mg g^{-1} dry wheat bran, which is equivalent to a 91.7% recovery of accessible sugar from the solid feedstock. For an optimal *E. coli* cultivation medium, we added essential metal salts such as $MgSO_4$, $CaCl_2$, and the M9 mineral salt mixture (see Experimental). *E. coli* cultivated for 24 h in the wheat bran-based hydrolysate medium standardized to 10 g glucose per L showed better growth rates and final cell densities compared to conventional complex media like Luria–Bertani (LB). This is most likely because of the higher amounts of sugars in the wheat bran-based growth medium. A parallel growth experiment with *E. coli* in LB medium supplemented with the same amount of glucose showed approximately equivalent growth rates and final OD_{600}. The results indicate that the chemical composition (*e.g.*, low lignin concentration) of wheat bran is particularly favorable to achieve efficient enzymatic hydrolysis with high fermentable sugar titers compared to other agricultural waste streams, such as corn stover[59] or wheat straw.[60] Additionally, it demonstrates the efficiency of the applied enzymatic hydrolysis protocol. The data shows that wheat bran constituents could replace commercial carbon and nitrogen sources, such as purified glucose or yeast extract. Therefore, the generated hydrolysate media can serve as an excellent replacement for commercially available cultivation media in industrial applications.

Technical scale-up with semi-continuous product capturing in 50 L controlled bioreactors

The economic feasibility of our CBT-ol production process is interdependent with end-product toxicity effects. The assays demonstrated that CBT-ol imparts almost no toxicity towards *E. coli* at concentrations of 2 g L^{-1}. Specific cell growth in cultivation media supplemented with increasing amounts of CBT-ol is comparable to the negative control (see ESI†). The lack of toxicity forms the basis for the efficient biotechnological production of CBT-ol *in E. coli*.

We aimed to optimize terpene product concentration by streamlining the entire bioprocess on a technically relevant scale of 50 L in controlled bioreactor systems. We utilized the wheat bran hydrolysate as fermentation medium in conjunction with the optimized CBT-ol production host. To facilitate the process, we additionally devised a comfortable capture method for parallel product removal. With regard to the latter, an adsorption-based bypass system was mounted on the bioreactor outlet, which enabled cyclization of the fermentation broth and semicontinuous depletion of the terpenoid product. In the developed system, the fermentation broth continuously flowed through an external column filled with hydrophobic Amberlite® XAD®-2 beads (Fig. 4A). The system provided for binding of hydrophobic products such as CBT-ol to the hydrophobic beads while the bacterial suspension was recycled into the fermentation process. This methodology reduced the product stress on the *E. coli* system and enabled simplified product recovery from the column material. For process optimization, it was essential to provide a sufficient flow rate in the column in order to minimize the residence time of the bacterial suspension within the bypass system as much as possible. This minimal residence time was essential to maintain a critical oxygen and nutrient supply for bacterial growth. The bypass adsorption system presented in this study has the potential to replace extremely time- and solvent-consuming methods such as liquid–liquid extraction, which are conventionally applied for the quantitative recovery of hydrophobic molecules from the reactor volume after fermentation. To maintain operations, we eluted adsorbed products from the hydrophobic resin at 24 h intervals with fresh ethyl acetate to keep capturing efficiency over the duration of the fermentation process.

Within this set-up, the CBT-ol production rate after the induction with IPTG (after 15 h, OD_{600} ~12) showed a constant correlation with the *E. coli* growth rate measured at OD_{600}

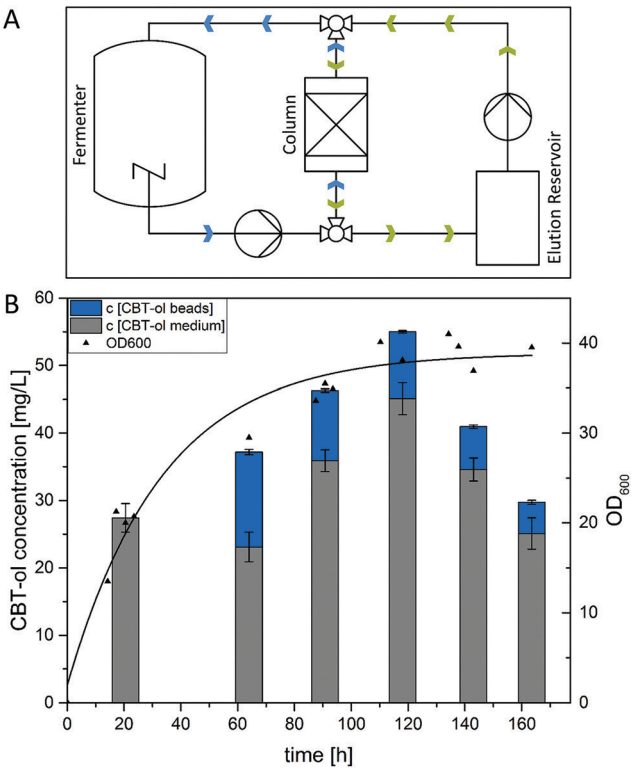

Fig. 4 [A] Sketch of the fermentation set up with the applied bypass system composed of two basic circuits. First circuit (blue arrows) serves to capture the product *via* absorption on the hydrophobic beads in the column. The second circuit (green arrows) is activated for the actual recovery and regeneration process by switching to a solvent which leads to product elution. The three-way valves allow an easy transition between the two circuits. [B] Bacterial growth curve and CBT-ol production rate during a 50 L fermentation with the applied bypass system. Extracted (blue) and remaining (gray) CBT-ol concentrations are shown for every point of measurement. The first capturing step started after 40 h and was then carried out every 24 h. The production dropped after 120 h which is therefore considered as the endpoint of the fermentation followed by continuous product depletion. The error bars represent the mean values ± standard deviation of technical triplicates.

(Fig. 4B). The presented data reveal that CBT-ol is not accumulated in the stationary phase and that the biosynthesis occurs mainly in the active growth phase. The terpene production rate dropped after 120 h and the overall concentration of CBT-ol in the bioreactor decreased with subsequent capturing steps. At this time, the fermentation process was terminated. Similar results were reported for the biotechnological production of other terpenoid compounds.[61,62] The recombinant CBT-ol biosynthesis is directly coupled and therefore interdependent with the endogenous FPP supply. FPP biosynthesis in *E. coli* is linked to an active cellular metabolism since it is essential for growth related respiratory quinone- and cell wall synthesis.[62,63] Therefore, the recombinant CBT-ol production closely follows exponential growth and ceases in the stationary phase. The cumulative CBT-ol concentration obtained within the 120 h fermentation time was measured to be 78.9 ± 2.4 mg L^{-1}. The specific CBT-ol productivity was 5.17 mg g^{-1} dry cell weight (DCW) and is therefore 2.9 times higher than in natural tobacco biomass (~0.18 mg g^{-1} dry weight[20]). In the 50 L reaction system, we could therefore generate a total CBT-ol quantity of 3.5 g. Based on this data, the terpene yield corresponded to ~3.4 mg CBT-ol per g glucose. The maximum and average production rates for CBT-ol were 1.48 ± 0.12 mg L^{-1} h^{-1} and 0.65 ± 0.02 mg L^{-1} h^{-1} over 120 h, respectively. With this method, it was possible to semi-continuously isolate more than 42% (w/w) of the generated CBT-ol from a large reactor volume already during the fermentation process and in a manageable time frame. A replacement of the adsorbent further increased the capturing efficiency subsequent to the fermentation. The pooled solvent fractions were concentrated *via* evaporation and subsequently separated with CPC.

To date, recovery of heterologous generated terpenes in almost all comparable studies relies on the *in situ* organic solvent extraction with dodecane or decane.[59,64,65] In comparison to alternative liquid–liquid extraction methods, this methodology prevents product volatilization into the gas phase and allows for significantly higher terpene titers, thus meeting all requirements of most literature studies.[66] However, this terpene recovery procedure is not trivial to implement on a larger scale and prevents simple downstream product recovery, since terpene products and the overlay solvent generally exhibit very similar physical properties. Therefore, an essential focus of this study was to establish a technically and economically scalable method for CBT-ol recovery and purification. In that regard, the developed bypass adsorption system enabled effective removal of toxic metabolites and separation of desired target products. Thereby, optimal *E. coli* growth and product recovery was achieved, while using less solvent compared to conventional methods.

With respect to the economic feasibility, most industrially relevant terpene production systems are currently designed for the generation of mono- or sesquiterpenes, such as limonene or nootkatone.[9,10,64,67] Especially in the field of low-cost biofuel production, the focus is exclusively on the less complex biosynthesis of smaller terpenoids.[59,64,67,68] In contrast, most diterpene-producing platforms are still less advanced than established production systems for mono- or sesquiterpenes. Insufficient precursor supply and flux into competing pathways as well as low expression of plant enzymes in microbial hosts are limiting factors that often prohibit high diterpene product titers.[69] Several years were spent optimizing specialized limonene production routes and processes[8] to achieve the current titers of 2.7 g L^{-1}.[9] The same effort in optimization of individual process steps, fermentation parameters and enzyme systems would lead to comparable diterpene titers in the gram scale. However, the target titer is also dependent on the pricing and application of the final product. In that respect, we estimate that titers of 500–1000 mg L^{-1} CBT-ol would be sufficient for industrial exploitation of our process, and we primarily envision to improve the cell densities (OD_{600} > 150) in technical scale fermentations.[70,71]

Purification and separation of cembratriene-ol isomers *via* CPC and chiral HPLC column

A major challenge in CBT-ol product purification and characterization is the separation of the respective isomers. In that regard, the concentrated crude extract, obtained in the capturing step, contained 10% (w/w) of racemic CBT-ol as well as other hydrophobic byproducts of the bacterial fermentation. To reduce costs and environmental impact, a purification procedure that provided high target product purity and recovery was favored. Centrifugal partition chromatography (CPC) allows versatile processing of different raw materials paired with reduced solvent consumption at high loading capacities.[40] In order to develop a CPC purification protocol for CBT-ol, we initially screened several biphasic solvent systems to find a system in which the partition coefficient (K) for racemic CBT-ol was within or close to the preferred "sweet spot" range ($0.4 < K < 2.5$). According to literature, this range provides a good compromise between the separation resolution, productivity, and solvent consumption in CPC.[72] In order to reduce experimental effort, the predictive thermodynamic model Conductor-like Screening Model for Realistic Solvation (COSMO-RS) was used as suggested by Hopmann *et al.*[73] For the selection of a suitable system, the partition coefficient K of CBT-ol was predicted in different biphasic solvent systems commonly used in CPC. For this study, only the molecular structures of CBT-ol and considered solvents were needed as input information. Solvent systems with various compositions and different polarities were screened, including hexane/ethyl acetate/methanol/water, heptane/ethyl acetate/methanol/water, butanol/methanol/water, hexane/ethyl acetate/acetonitrile and hexane/ethanol/acetonitrile. Based on the COSMO-RS predicted K-values of CBT-ol, the hexane/ethanol/acetonitrile system was selected to be most suitable. Systems with ethyl acetate and a K value of CBT-ol in the preferred range were excluded in order to avoid any UV-interference with the CBT-ol absorbance spectrum at the point of detection at 210 nm. Subsequently, the hexane/ethanol/acetonitrile system was experimentally evaluated to verify the predicted partition coefficients of CBT-ol and determine the K values of the unknown impurities, which could not be taken

into account in the COSMO-RS screening study due to missing molecular structures. Based on the K values determined by shake flask experiments, an acetonitrile/ethanol/n-hexane system composition of 27.4/1.6/71 (v/v/v) was selected as a suitable system for CBT-ol separation with CPC. The CPC separation was performed in descending mode, using the lower phase of the solvent system as the mobile phase. A stationary phase retention (S_F) of 0.63 was obtained. After the sample injection, only a small stationary phase loss of about 4 mL was detected over the entire runtime. Fractions were collected every 30 s and analyzed by GC-FID. In Fig. 5A, the reconstructed CPC chromatogram is presented. As apparent from the chromatogram, a small peak overlap occurred. Still, sufficient separation of racemic CBT-ol from main impurities was achieved with this system.

As a good compromise between the CBT-ol purity and recovery, the fractions were combined in a way that both exceeded 95% (Fig. 5A). According to the CPC chromatogram, a purity of racemic CBT-ol higher than 99.5% with a 28.3% loss of the target product would be possible. A productivity of 390 mg h^{-1} with a solvent consumption of 1.23 mL mg^{-1} was obtained, calculated based on a mass load of 280 mg per pulse injection and a purity of racemic CBT-ol of 95%.

The subsequent separation of α- and β-CBT-ol isomers was accomplished *via* a chiral HPLC column at a semi-preparative scale. The chromatogram indicated two clearly separated compounds (Fig. 5B), which could be identified as the respective CBT-ol isomers with NMR analysis (see ESI†). The ^{13}C and ^{1}H ($CDCl_3$) NMR data matched the reported data sets for thunbergol and 4-epiisocembrol, respectively (Table 1). Signature NMR signals like the ^{13}C NMR singlet at δ 72.8 and 73.8 for the C-4 atom connected to the hydroxyl group and the ^{1}H singlet at δ 1.34 and 1.27 (C-18 methyl group) could be confirmed.[28] The peak area integration revealed a relative ratio of 1 : 2.3, α- and β-CBT-ol in the racemic mixture, respectively. These results contrast with the isomer distribution reported *in planta*, where the α-CBT-ol isomer was dominant.[20]

Table 1 ^{13}C chemical shifts ($CDCl_3$) and assignments for α- and β-CBT-ol compared to the literature[28]

C	α	Ref.	β	Ref.
1	46.1	45.9	46.3	46.2
2	129.3	129.0	127.1	126.9
3	138.2	138.3	138.9	138.9
4	72.8	72.5	73.9	73.8
5	43.1	43.1	44.2	44.1
6	22.7	22.6	23.6	23.5
7	128.6	128.6	127.9	127.9
8	132.5	132.2	132.7	132.4
9	36.9	36.8	37.0	36.9
10	23.8	23.8	23.8	23.7
11	125.3	125.2	125.0	124.9
12	132.6	132.3	133.0	132.7
13	39.3	39.2	39.2	39.1
14	27.7	27.6	28.1	28.0
15	33.0	33.0	33.2	33.1
16	20.6	20.4	20.8	20.7
17	19.6	19.5	19.5	19.4
18	28.2	28.1	29.5	29.3
19	14.9	14.7	15.0	14.8
20	15.2	15.0	15.3	15.1

Assessment of insect repellent activity by a specific aphid colonization test

CBT-ol is reported to exert an insect protective effect *in planta*.[32] Most interestingly, genetic plant modifications leading to higher CBT-ol concentration enhance the insect protective effect.[20] These reports lead to the expectation that recombinantly generated CBT-ol could be applied as an insect repellent on plant material. To test this hypothesis, a two-choice aphid colonization experiment was carried out, comparing CBT-ol-treated plants and untreated controls upon release

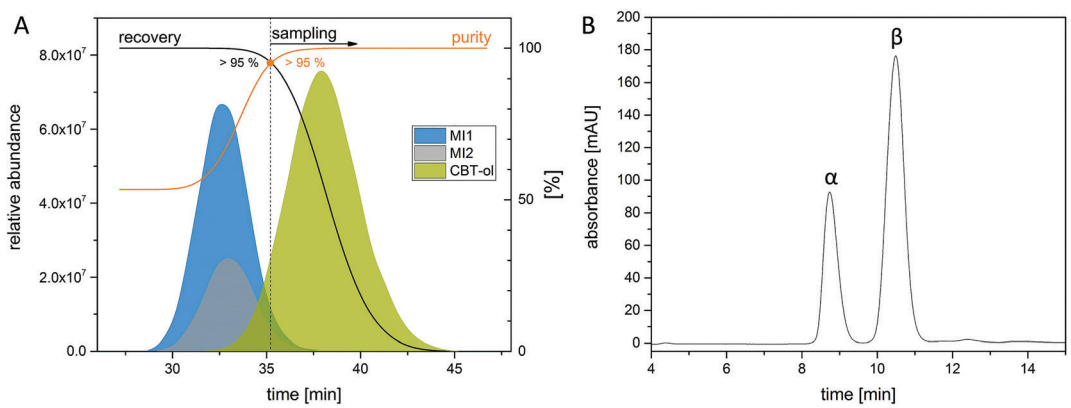

Fig. 5 [A] Chromatogram of a CPC batch separation of racemic CBT-ol from crude extract using the solvent system acetonitrile/ethanol/n-hexane 27.4/1.6/71 (v/v/v) (descending mode: lower phase as mobile phase, 8 mL min^{-1} mobile phase flow rate, 1700 rpm, m_{inj} = 280 mg, V_{inj} = 2 mL). Fraction content was determined and quantified *via* GC-FID. High purity as well as recovery rates were intended to separate CBT-ol (green peak) from the major impurities (blue and gray peaks). A purity >95% was aspired choosing a specific starting point for the sample fractioning after 35.2 minutes. [B] Separation of the two CBT-ol isomer forms using a chiral Lux® 5 μm amylose-1 LC column 250 × 10 mm (mobile phase: ACN and H_2O (7 : 3), 2.2 mL min^{-1} flow rate, m_{inj} = 40 mg, V_{inj} = 2 mL) resulted in a chromatogram with two clear and independent peaks representing the α- and β-CBT-ol (ratio 1 : 2.3).

of live aphids (*Rhopalosiphum padi*). We observed an increased aphid deterrent activity regarding the infestation rate for the CBT-ol-treated plants. This protective effect reached a maximum of 70% within the first 48 h after CBT-ol racemate application (Fig. 6A). We subjected the data set to a Student's *t*-test, which indicated a high significance ($p < 0.005$) with regard to the CBT-ol aphid repellent activity. These results are consistent with the previous reports indicating that increased CBT-ol content in plants results in lower aphid colonization,[20] and potentially acting as a contact insecticide.[32] To gain a more detailed insight into the bioactivity of the two CBT-ol isomers, we investigated the specific aphid deterrent effects for each compound separately. The data initially suggested a slightly higher activity of the α-isomer, but a statistical evaluation did not support a significant difference between the CBT-ol isomer activities. In the control experiments with commercially available plant-derived and pyrethrin-based insecticide,[74] all aphids that initially colonized the assay plants were killed. By contrast, the application of either CBT-ol isomer only induced an avoidance behavior in the applied aphids, which indicates that CBT-ol has a deterrent but no actual toxic activity toward these pests. This suggests that CBT-ol utilization may mimic the biological protective mechanism of the tobacco plant and exert no toxic effects on beneficial insects, such as bees, which would significantly benefit agricultural efficiency *per se*.

Regarding CBT-ol formulation, application and dosage as an insecticidal repellent, further studies have to be carried out. In summary, the data suggest that CBT-ol may play a promising role in future plant protection strategies as a deterrent. Additionally, the application of CBT-ol as a topical insect repellent for human use may be an alternative. In that respect, the development of a cosmetic application may be associated with reduced regulatory demands and could accelerate market entry for this interesting compound.

Extended cembratriene-ol bioactivity studies

Due to previous literature evidence, this study initially focused on the characterization of CBT-ol as an insecticide deterrent. Although various hydroxylated cembrenes have been reported to show neuroprotective[26] and anti-tumor[75] activities, at present no data have been reported for the monohydroxylated CBT-ol in this regard. To elucidate whether the CBT-ol racemate has anti-microbial, anti-tumor or extended anti-insecticidal activities, we conducted an array of *in vitro* toxicity tests with respective cell lines. We applied the purified CBT-ol racemate to nine different cell types including human cancer cells (MCF-7, HeLa), bacteria (*E. coli*, *B. subtilis*, *M. luteus*), yeast (*C. glabrata*, *S. pombe*, *Z. rouxii*) and *Spodoptera frugiperda* insect (*Sf21*) cells. Of the nine tested cell lines, six showed growth inhibition when exposed to CBT-ol. In that respect, the half maximum inhibitory concentration (IC_{50}) could be calculated (Fig. 6B). *S. pombe*, *B. subtilis and M. luteus* appeared to be the most sensitive organism to the CBT-ol treatment with the lowest IC_{50} values of 8, 9 and 10 μM, respectively. By contrast, the human breast adenocarcinoma and human cervix carcinoma cell lines MCF-7 and HeLa as well as the insect cell *Sf21* were more resilient toward CBT-ol exposure, with IC_{50} values of 42 μM, 51 μM and 68 μM, respectively. No CBT-ol toxicity effects were observed for *E. coli*, *Z. rouxii* and *C. glabrata*.

The relatively low CBT-ol toxicity toward insect cells may support its observed action as an insect repellent and not as an insecticide. Furthermore, the enhanced CBT-ol sensitivity of Gram-positive bacteria (*B. subtilis* and *M. luteus*) might be attributed to the different cell wall composition compared to Gram-negative bacteria, such as *E. coli*. While the Gram-negative cell wall is rather impermeable to lipophilic molecules,[76] the hydrophobic nature of CBT-ol may be better suited to enter Gram-positive bacterial cells. This selective activity may trigger the development of CBT-ol as a specific antibiotic or topical

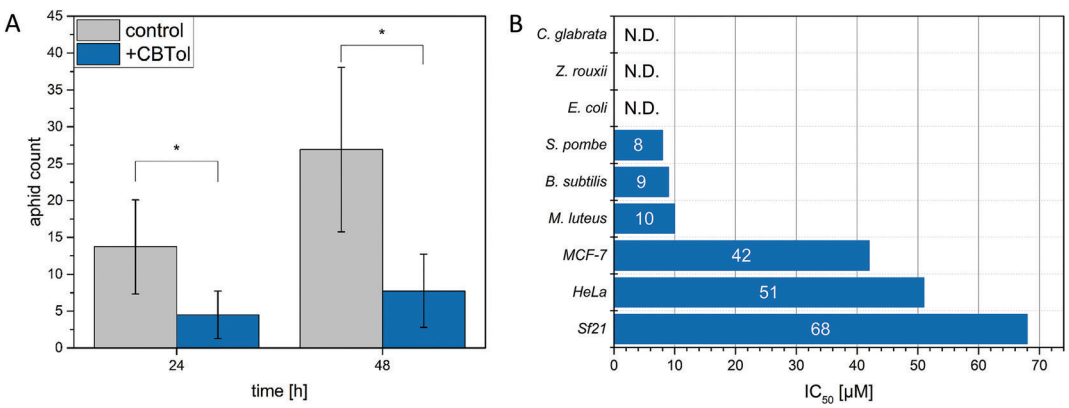

Fig. 6 [A] Biological effect of a 0.25% CBT-ol solution on the colonization behavior of aphids. Treated plants (blue) are compared to a control group (gray), showing that the aphid count was reduced by up to 70%. The results are represented as the mean ± standard deviation of multiple repeated experiments (*$p < 0.005$). [B] Effect of CBT-ol on different cell lines and types represented as the particular IC_{50} values. All experiments were based on a 6-fold approach. The experiments on the cell lines (HeLa; MCF-7; *Sf-21*) were performed two times to calculate the IC_{50} values (N.D.: IC_{50} were not determined due to no measurable activity).

antiseptic preparation against clinically relevant pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Listeria monocytogenes*.[77,78]

Experimental

General

Components for cultivation media were obtained from Roth chemicals and Applichem GmbH. Extraction was performed with technical grade ethyl acetate and hexane from Westfalen AG. For all other procedures, the highest purity grade chemicals were used. Acetic acid, acetonitrile, ethyl acetate, ethanol and hexane were obtained from Roth chemicals. $CDCl_3$ was purchased from Sigma Aldrich. Cellic® HTec and Cellic® CTec for enzymatic hydrolysis were obtained from Novozymes and α-amylase (*Bazillus licheniformis*) from Sigma Aldrich. Phusion polymerase for polymerase chain reactions (PCR), restriction enzymes and T4 Ligase were purchased from Thermo Fisher Scientific. DNA preparations kits (Thermo Fisher Scientific) and gel extractions kits (Analytik Jena) were used for DNA preparation.

Cloning

E. coli HMS 174 (DE3) was used for cloning and was cultivated at 37 °C in lysogeny broth (LB) medium. Chloramphenicol (34 μg L^{-1}) and kanamycin (50 μg L^{-1}) were added as required. All primers and genes were synthesized by Eurofins Genomics. Standard protocols were used for PCR and ligation. The RBS calculator software tool[49,51] was used to design and evaluate the relevant ribosomal binding sites (RBS). The following genes were used in this study: isopentenyl pyrophosphate isomerase (idi) from *Haematococcus lacustris* (GenBank: AAC32208.1); geranylgeranyl pyrophosphate synthetase (crtE) from *Pantoea ananatis* (GenBank: ADD79325.1); Terpene Cyclase/Synthase (TPS) from *Nicotiana sylvestris* (GenBank: AAS46038.1); 1-deoxy-D-xylulose-5-phosphate synthase (dxs) from *Escherichia coli* (GenBank: AF035440.1).

All genes were custom synthesized and codon optimized for best possible expression results. All vectors, genes and RBS sequences were verified by sequencing (Eurofins Genomics). The iterative assembly of the synthetic operon receiving a combinatorial library was performed according to the BioBrick principles[53] described by Zelcbuch *et al.*[49] The construction of the synthetic operon was performed in pNiv vectors. The whole operon library was transferred into a pSB4K5 expression vector for production studies. Idi, crtE, dxs, pSB4K5 and pNiv were kindly provided by prof. R. Milo, Weizmann Institute of Science, Israel.

CBT-ol *in vivo* production

75 mL of M9 Minimal Medium supplied with 4 g L^{-1} glucose, 1 mg L^{-1} thiamine and 1 mg L^{-1} biotin containing the required antibiotic in a 250 mL baffled shake flask was inoculated with 1 mL of overnight LB culture of pre-engineered *E. coli* harboring the appropriate plasmid. The cultures were grown at 37 °C at 130 rpm up to an optical density (OD_{600}) of 0.5–0.7 before inducing with IPTG (150 μM) and switching to 22 °C for 2 days.

CBT-ol isolation for analytical purposes

For analytical CBT-ol isolation within the screening process, 30 mL of the selected cultures was lysed by brief sonication. Then, 15 mL of a mixture of ethyl acetate and hexane (1 : 1) was added. The suspension was then strongly shaken for 30 min. A centrifugation step at 8000*g* for 60 s was carried out to separate the phases. A defined volume of the upper organic phase was then sampled and evaporated under a gentle N_2 stream. The residues were resuspended in 1 mL of ethyl acetate and analyzed.

CG-MS and NMR analytics

Extracted terpene products were analyzed by a Trace GC Ultra with DSQII (Thermo Scientific). The sample was loaded by TriPlus AS onto an SGE BPX5 column (30 m, I.D. 0.25 mm, film 0.25 μm). The initial column temperature was set to 50 °C and was maintained for 2.5 min before a temperature gradient of 10 °C min^{-1} to 320 °C was applied. The final temperature was kept for additional 3 min. MS data were recorded at 70 eV (EI) and *m*/*z* (rel. intensity in %) as total ion current (TIC). The data were collected in full scan mode (*m*/*z* 50–650). Terpenes were identified by comparison of GC-MS retention times and mass spectra to a commercially available CBT-ol standard (Biomol) and mass spectra data of the NIST Standard Reference Database. Concentrations were quantified correlating the FID peak area to a defined CBT-ol standard of known quantity, ranging from 85 ng to 2.5 mg (see ESI†).

The NMR spectra were recorded in $CDCl_3$ with a Bruker Ascend™ 400 MHz NMR spectrometer. All chemical shifts are relative to $CDCl_3$ at δ = 7.26 (^{1}H-NMR) or $CDCl_3$ at δ = 77.16 (^{13}C-NMR) using the standard δ notation in parts per million.

Wheat bran hydrolysation and media preparation

For the preparation of 1 L of 10% (w/v) biomass hydrolysate solution, 500 mL of 50 mM sodium acetate buffer (pH 5 adjusted with acetic acid) was mixed with 100 g of wheat bran and supplemented with 100 μL of amylase. After incubation for 1 h, the mixture was autoclaved at 121 °C for 15 min. Subsequently, 1% of Cellic® HTec and Cellic® CTec, respectively, were solved in another 470 mL of sodium acetate buffer and centrifuged to separate sediments before sterile filtration with a 0.2 μm filter. The enzyme mixture was added to the wheat bran mash for a final volume of 1 L. After 72 h of hydrolysis at 50 °C and moderate constant shaking, the solid residue was removed using filter paper followed by a sterile filtration step of the liquid phase. For the large scale hydrolysation, 5 kg of wheat bran was added to 35 liters of sodium acetate buffer and transferred to an LP 75 L Bioreactor (Bioengineering). Starch hydrolysis with 5 mL of amylase for one hour at 37 °C was followed by sterilization at 121 °C for 30 minutes with constant stirring. For the enzymatic hydrolysis, 3.0 volume percent (1.5 L) of each enzyme were mixed with the same amount of hydrolysis buffer and pumped directly into the reactor using a

sterile filter (final volume 45 L). The reactor temperature was reduced to 50 °C with constant stirring for 48 hours and a pH of 5. To clarify the hydrolysate, a plate separator was used at 9000 rpm followed by a final sterile filtration step. The final hydrolysate media was based on modified M9 minimal media with a final glucose concentration of 10 g L^{-1} and a pH of 7.

Hydrolysate media

M9 salts mixture	50 mL
60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, 1 L H_2O (pH 7.4)	
1 M $MgSO_4$	250 μL
1 M $CaCl_2$	100 μL
Hydrolysate (10%)	250 mL
Kanamycin monosulfate	50 mg
H_2O	Up to 1 L

Fed-batch fermentation including bypass system

The large-scale fermentation was performed in a 75 L Bioreactor (Bioengineering). Initial cultivation volume was set to 32 L using optimized hydrolysate medium (10 g L^{-1} glucose). The pH was set to 7.0 and controlled by 25% ammonium hydroxide and 25% phosphoric acid. We used 2 L of an 8 h culture grown in LB medium for inoculation (final OD_{600} ~0.1). The feed solution consisted of pure hydrolysate solution (~40 g L^{-1} glucose). The feed protocol started with 1.5 mL min^{-1} and was increased to 3 mL min^{-1} after 40 h. The total volume of consumed feed solution was 18 L within 120 h. Cultivation temperature was kept constant at 37 °C for the initial growth phase and was reduced to 22 °C after induction with 1 mM IPTG at an OD_{600} of approximately 12. Dissolved oxygen was controlled at 40% air saturation by adjusting the agitation rate (300–530 rpm) at a constant airflow of 10 standard liters per minute of compressed air. Cell density and product output were monitored by periodic sampling.

For the semicontinuous product isolation during the fermentation, a 2 L chromatography column (10 × 24 cm) was applied and filled with 1 kg Amberlite® XAD®-2 beads (Sigma-Aldrich). The whole column was sterilized with 1 M NaOH for three hours and washed with sterile water afterward. The bypass system was mounted to the reactor *via* hose pumps and frits (45 μm pore diameter). The entire reactor volume was constantly pumped through the packed column at a flow rate of 120 mL min^{-1} to prevent blockage. Every 24 hours, the column was washed with 8 L demineralized water followed by a product elution step with 1.5 L ethyl acetate. After a final wash with sterile water (10 L) and sterile air, the column was again incorporated into the fermentation circuit. Ethyl acetate from all elution steps was pooled and evaporated using a rotary evaporator (40 °C, 240 mbar) and the concentrated crude extract was stored at −20 °C.

Terpene purification by CPC

Experiments were performed at room temperature in an SCPC 250 unit from Armen Instrument (now called CPC 250, Gilson Purification SAS, Saint-Avé, France). The unit consists of two single columns connected in series with a total column volume of 182 mL. The maximum achievable rotational speed is 3000 rpm and the whole column can be operated at a maximum pressure drop of 100 bar. The CPC unit was connected to two isocratic preparative HPLC pumps with maximum flowrates of 50 mL min^{-1}, one for filling the column with the stationary phase and the other one for pumping the mobile phase during the separation. The effluent was monitored with a UV detector (ECOM DAD600 2WL 200–600 nm, Prague, Czech Republic) at 210 nm, and a fraction collector (LS 5600, Armen Instrument, France) was used for collecting fractions during the separation. The feed sample was introduced through a six-port manual injection valve. An injection loop of 2 mL was used.

We used the biphasic solvent system acetonitrile/ethanol/*n*-hexane 27.4/1.6/71 (v/v/v) to separate the concentrated CBT-ol crude extract. The biphasic system was prepared by mixing the respective volume portions of the solvents at room temperature. The mixture was vigorously shaken and equilibrated at room temperature for at least two hours. Afterwards, the two phases were filled in a separation funnel, split and conveyed into two distinct reservoirs. Before use, the phases were degassed in an ultrasonic bath. The lower phase was used as the mobile phase in descending mode. The feed mixture was prepared by dissolving the concentrated CBT-ol crude extract obtained from the bypass adsorptive isolation in the mobile phase (140 mg mL^{-1}). At the beginning of the CPC experiment, the columns were filled with the stationary phase (upper phase) at a flow rate of 40 mL min^{-1}. Afterwards, the rotational speed was set to 1700 rpm and the mobile phase was pumped through the column at 8 mL min^{-1} until no more stationary phase eluted from the column. Then, 2 ml of the feed mixture was injected, the effluent was monitored at a wavelength of 210 nm and fractions were collected every 30 seconds. The CBT-ol-containing fractions were pooled and evaporated under a N_2 stream. The purified racemic CBT-ol oil was stored at −20 °C.

Semi-preparative CBT-ol isomer separation

The two isomers α- and β-CBT-ol were separated *via* High-performance liquid chromatography (HPLC: UltiMate™ HPG-3200BX pump, UltiMate™ 3000 AFC and DAD from Thermo Fischer; Jetstream II Plus Column Thermostat; Degasser from Knauer) using a Lux® 5 μm amylose-1, LC column 250 × 10 mm (Phenomenex) and a diode array UV detector. The separation was carried out in an isocratic mode with a mobile phase composed of ACN and H_2O (7 : 3) (2.2 mL min^{-1}, 50 °C, 15 min) with active peak fraction collection. The injection volume was 2 mL with a concentration of 20 mg mL^{-1} of CPC-purified racemic CBT-ol oil in ACN and H_2O (7 : 3). The chromatogram was recorded at 210 nm wavelength. The corresponding fractions with the separated products were pooled and evaporated under a N_2 stream. The pure isomers were stored at −20 °C for further usage.

Bio-insecticide assay

The aphid response to the isolated and purified compound was measured in a two-choice colonization experiment. The compound was applied as 0.25% solutions in ethyl acetate to separated groups of wheat seedlings. The plants were grown in the laboratory at RT in a homemade growing chamber for 7 days on cotton wool. A nebulizer was used for the equal application of the solution. Control seedlings were treated with pure ethyl acetate. The solvent had evaporated completely after 1 hour, and all plants were placed randomly in a test box and the aphids (*Rhopalosiphum padi*) were distributed evenly at a certain distance to all plants. Aphids that settled on the green parts of each group were counted after 24 h and 48 h. The results were statistically analyzed using Student's *t*-test.

Conclusions

We strongly advocate the substitution of non-target discriminant insecticides (*e.g.*, imidacloprid, thiamethoxan and clothianidin) with bio-degradable insect deterrents, such as CBT-ol. The move towards biological insect deterrents represents a fundamental change in crop protection strategies, which would support and expand sustainable agricultural practices. Specifically, we would like to propose that future crop protection methods focus on deterrent rather than lethal active ingredients to protect beneficial and increasingly endangered insect populations, which are essential for pollination of agricultural crops. In this context, the EU commission has recently decided to significantly limit the applicability of toxic insecticides, particularly of the neonicotinamide family in order to enhance protection of bee populations.[17–19]

In this study, we present the heterologous production of the complex diterpene CBT-ol in an engineered *E. coli* production strain. Application of combinatorial RBS adaptation to each biosynthetic bottleneck enzyme provided for an improved protein expression and metabolic flux in the host cell. Moreover, we devised a commercial enzyme system to convert a wheat bran-based milling waste stream into a highly effective *E. coli* fermentation medium. On this waste stream-based growth medium, we obtained CBT-ol titers of 78.9 mg L^{-1} using a technically relevant 50 L bioreactor. To avoid toxic end-product effects on *E. coli* and enable in-process target compound enrichment, we devised an adsorption-based bypass system, which was directly connected to the bioreactor system. We developed a centrifugal partition chromatography (CPC)-based CBT-ol purification strategy that allowed for 95% purity and recovery of the target compound to work up the crude extract eluted from the bypass absorbent. Moreover, this CPC strategy allowed for the reduced use of organic solvents during purification, which rendered the process environmentally benign. To our knowledge, this is the first account of a CPC strategy for the purification of a microbial-generated diterpene. The CBT-ol methodologies disclosed here are entirely scalable from the laboratory to the technical scale, which provides a direct route for industrial exploitation.[79] The application of chiral HPLC chromatography allowed for the isolation of CBT-ol α- and β-isomers. For the first time, we could demonstrate that the CBT-ol isomer distribution in heterologous *E. coli* systems contrasts the situation in plant systems. Initial aphid avoidance assays indicated that both α- and β-CBT-ol isomers had equivalent effects, demonstrating insect repellent activity. It was confirmed in *in vitro* assays with an insect cell line that CBT-ol was not directly toxic. Therefore, we suggest that CBT-ol has repellent and not direct insecticidal activity. This insect repellent activity may be useful in a cosmetic preparation and is also of particular importance for agricultural applications, as beneficial insects such as bees are not affected by its application. In addition, CBT-ol does not accumulate[34] in soil or aquatic habitats, which contrasts the situation with synthetic insecticides such as neonicotinoids. Most interestingly, we conducted extended cellular toxicity assays including yeast, bacteria and human cancer cell lines and for the first time, we can report that the CBT-ol racemate shows selective toxicity toward Gram-positive bacteria. This observation may trigger further developments of CBT-ol as a selective antibiotic or topical antiseptic toward clinically highly relevant pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Listeria monocytogenes*.[78]

With reference to other reports concerning transfer of laboratory data to industrially applicable processes, starting from a CBT-ol titer of 78.9 mg L^{-1} represents a manageable leap to a commercially viable process in the g L^{-1} scale.[8,80] Currently, genomic integration is frequently reported to enhance both metabolic flux and microbial host productivities in non-terpenoid production systems.[81] However, particularly the integration of plasmid-optimized operons for heterologous terpenoid pathways can result in unpredictable metabolic effects that negatively affect both, protein expression and product titer.[82] Therefore, we would suggest to address other system improvements, such as the evaluation and adaption of different microbial production chassis[70,83] as well as the iterative optimization of the fermentation process towards higher cell densities.[70,71]

Conflicts of interest

There are no conflicts to declare.

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4 From microbial upcycling to biology-oriented synthesis: combining whole-cell production and chemo-enzymatic functionalization for sustainable taxanoid delivery

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4.1. AUTHORS' CONTRIBUTIONS

MAX HIRTE: General project conception, coordination, experimental planning and conduction. This included strain engineering, process development, product analysis and screening of suitable solvent systems for diterpene separation.

WOLFGANG MISCHKO: Significant and comprehensive support as well as consulting during the entire project. In particular the establishment of fundamental methods, processes and analytics for heterologous terpene production and characterization as well as the development of suitable solvent systems for diterpene separation.

KATARINA KEMPER: Support in terpene-related aspects and concerns.

SIMON RÖHRER: Execution and maintenance of the CPC-based purification system. Cooperation in screening and developing the suitable biphasic solvent mixture for taxadiene separation.

CLAUDIA HUBER: Support in elucidation of terpene structures.

MONIKA FUCHS: Coordination, comprehensive support and supervision of the research project.

WOLFGANG EISENREICH: Support in elucidation of terpene structures as well as critical revision of the manuscript.

MIRJANA MINCEVA: Supervision of the CPC-based product purification as well as critical revision of the manuscript.

THOMAS B. BRÜCK: Drafting of the research objectives, project lead and cooperation in writing the manuscript as well as its critical revision.

4.2. SUMMARY

This study summarizes the development of a new, holistic, fast and sustainable strategy for the biotechnological production and purification of the structurally complex diterpene taxadiene and a subsequent chemo-enzymatic functionalization. Taxadiene represents the macrocyclic core of the clinically relevant antitumor agent paclitaxel, which is still very challenging to produce on an industrially relevant scale.

As a starting point for general heterologous terpene generation, an isoprenoid production operon was optimized and harmonized with regard to carbon flux and product yield. For this purpose, an optical read out system based on the carotenoid lycopene was developed. Compared to conventional inducible taxadiene production platforms, the obtained constitutive microbial systems demonstrated improved characteristics concerning yield, reproducibility and transferability. Utilizing this engineered *E. coli* strain, production of 364.4 ± 10.7 mg/L taxadiene was possible within 44 h.

In parallel, the microbial recycling and transformation of industrial waste streams, such as corn steep liquor as by-product in the wet corn milling process, was demonstrated for sustainable taxadiene production.

In order to improve the supply of purified taxanoid molecules, different terpene specific extraction methods were evaluated. In this context a fast and simple two-step process for the isolation from *E. coli* high cell density cultures was developed and optimized. Subsequently, centrifugal partition chromatography (CPC) was evaluated as suitable method for diterpene purification. This new downstream processing strategy delivered 249.0 ± 11.1 mg/L taxadiene with a purity of 95% and represents an efficient alternative to conventional biphasic, *in situ* extraction and purification methods. The resulting taxadiene was further functionalized by an *in vitro* lipase (CalB)-mediated epoxidation reaction as an efficient way to activate carbon bonds in diterpenes. The highly specific and mass efficient production of taxa-4(5),11(12)-bisepoxide with an enantiomeric excess of over 83% was achieved. This bis-epoxilated taxanoid molecule represents the basis for further derivatizations by various synthetic strategies. As a fundamental concept, all these investigated production-, purification- and functionalization steps are designed for the transfer into commercially relevant scales.

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From microbial upcycling to biology-oriented synthesis: combining whole-cell production and chemo-enzymatic functionalization for sustainable taxanoid delivery†

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A holistic bio-process based on the microbial upcycling of low-value feedstocks and leading to chemo-enzymatically derived, functionalized taxanoids was established. The upcycling of biotechnological by-product streams glycerol and corn steep liquor by an engineered *Escherichia coli* strain that constitutively expressed bottleneck enzymes within the MEP-pathway led to the formation of 364.4 ± 10.7 mg L^{-1} taxadiene within 44 h. In contrast to standard inducible systems, our constitutive microbial production system provided concomitant growth and taxadiene formation. This strategy is the basis for subsequent continuous taxadiene production processes, which is favorable under economic constraints. The growth dependent taxadiene production showed improved yields, reproducibility and transferability at any scale compared to inducible taxadiene production platforms examined in this study. Additionally, we developed new taxadiene isolation and purification strategies. To that end, these new downstream processing strategies display efficient alternatives to conventional biphasic, *in situ* extraction and purification procedures. Specifically, we developed a rapid and easy two-step extraction procedure followed by centrifugal partition chromatography purification. This process strategy provided 249.0 ± 11.1 mg L^{-1} taxadiene with 95% purity from *E. coli* high cell-density cultures at a liter scale. To functionalize taxadiene, a mild lipase mediated epoxidation reaction was devised. Optimization of this biology-oriented synthesis strategy led to a quantitative conversion of taxadiene to taxa-4(5),11(12)-bisepoxide with an enantiomeric excess of over 83%. The holistic process strategy afforded in excess of 215 mg taxa-4(5),11(12)-bisepoxide by upcycling the low-value feedstocks glycerol (300 g) and corn steep liquor (25 g).

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Introduction

Legislative measures for reducing CO_2 emissions, and price fluctuations in fossil fuel resources, drive the development and industrial deployment of sustainable bio-based processes. These sustainable processes should comply with the 12 principles of green chemistry.[1,2] Consequently, by-product streams, such as glycerol from bio-diesel production or corn steep liquor (CSL) from wet corn milling, represent optimal carbon and nitrogen feedstocks for designing new bio-processes. One particularly resourceful option for such a process is the use of metabolically engineered whole-cell biocatalysts, such as *Escherichia coli*, which can convert these residual feedstocks into high-value target compounds.[3–6] Additionally, this "microbial upcycling" processing strategy complies with the aim of a zero waste, circular bio-economy.[7]

The family of diterpenoid natural products is structurally and functionally highly diverse. It encompasses several high-value compounds such as the tumor therapeutic Paclitaxel or the fragrance precursor Sclareol.[8,9] Due to their structural complexity and predominantly low natural abundance, synthetic or semi-synthetic production routes are often applied for their industrial-scale production.[10,11] However, synthesis of these compounds is often associated with low product yields and/or significant generation of toxic waste streams.[12,13] Hence, a biotechnological production route represents an attractive and alternative strategy to chemical synthesis.[14–22] In this respect, engineered microbial platforms provide a technically relevant supply of the universal terpene precursors isopentenyl dipho-

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sphate and dimethylallyl diphosphate, which in conjunction with expression of prenyl transferases and diterpene synthases, facilitate the production of diterpenes.[23–25] To date, recovery of these heterologously generated diterpenes has almost exclusively relied on organic solvent-mediated *in situ* extraction mediated by a dodecane or decane overlay.[26–28] In comparison with post-cultivation liquid–liquid extraction, this methodology yields significantly higher achievable diterpene titers that can be obtained from 1 L of cell culture.[29,30] However, the applicability of *in situ* extraction method for the diterpene molecules has to be controlled in terms of further downstream recovery, synthetic work-up options and their associated costs. Additionally, the *in situ* extraction method faces the issue of premature compound extraction if *in vivo* functionalization of the diterpene molecule is to be conducted. Furthermore, such diterpene-centered *in vivo* catalyzed transformations often suffer from low enzyme activity or promiscuity. Moreover, product instability and toxicity effect towards to the microbial production system are observed, leading to low target product yields.[31–34] However, functionalization of the diterpene skeleton is mostly required to convey biological activity.[18,35–37]

Therefore, new routes for efficient diterpene recovery and functionalization have to be identified to enable eco-friendly diterpenoid production at economically relevant scales. One production strategy is the initial biotechnological production of a diterpene scaffold combined with subsequent *in vitro* functionalization. In general, such biology-oriented strategies are highly promising in the identification of new lead compounds.[38–40]

One of the most prominent and structurally complex diterpenoids is the clinically relevant anti-tumor agent Paclitaxel.[41] In this regard, the bio-production of the macrocyclic core, which comprises taxadiene (TD) and its hydroxylated congeners (taxanoids), remains challenging to realize at large scales.[14,27,34] In this study, we report a new holistic approach for the sustainable delivery of promising taxanoid diterpenoids that are targeted at pharmaceutical applications. In this context, we demonstrate the possibility of upcycling by-product streams of industrial origin by utilizing glycerol and CSL for the sustainable production of the diterpene TD. We initially engineered *E. coli* strains to express enzyme complements that are required for TD generation. We identified that constitutive expression of those enzymes improve TD yields substantially. Biphasic, *in situ* extraction by the overlay of dodecane was compared to a two-step post-cultivation liquid–liquid extraction. Subsequently, a new purification method for TD enrichment and recovery from high-cell density cultures was established. This demonstrates that terpenoids such as TD can be efficiently purified from cell broth-derived extracts in a single step using centrifugal partition chromatography (CPC). Additionally, a highly specific biology-oriented synthesis approach for purified TD is reported that allows for specific diterpene scaffold functionalization. More specifically, a mild lipase-mediated epoxidation that follows the Prilezahev reaction was established.[42] This reaction provides mass efficient access to a single bis-epoxilated taxanoid molecule that can be further derivatized by various, synthetic strategies.

Results and discussion

Strain engineering

Heterologous production of diterpenes have been conducted in several organisms such as plants, fungi, yeast and bacteria.[43–45] In that regard, *S. cerevisae* and *E. coli* are widely used because they are well studied, easy to manipulate and fast to cultivate. In this study, we choose *E. coli* as production host, as higher diterpene production titers are achievable in shorter time compared to alternative production systems.[21]

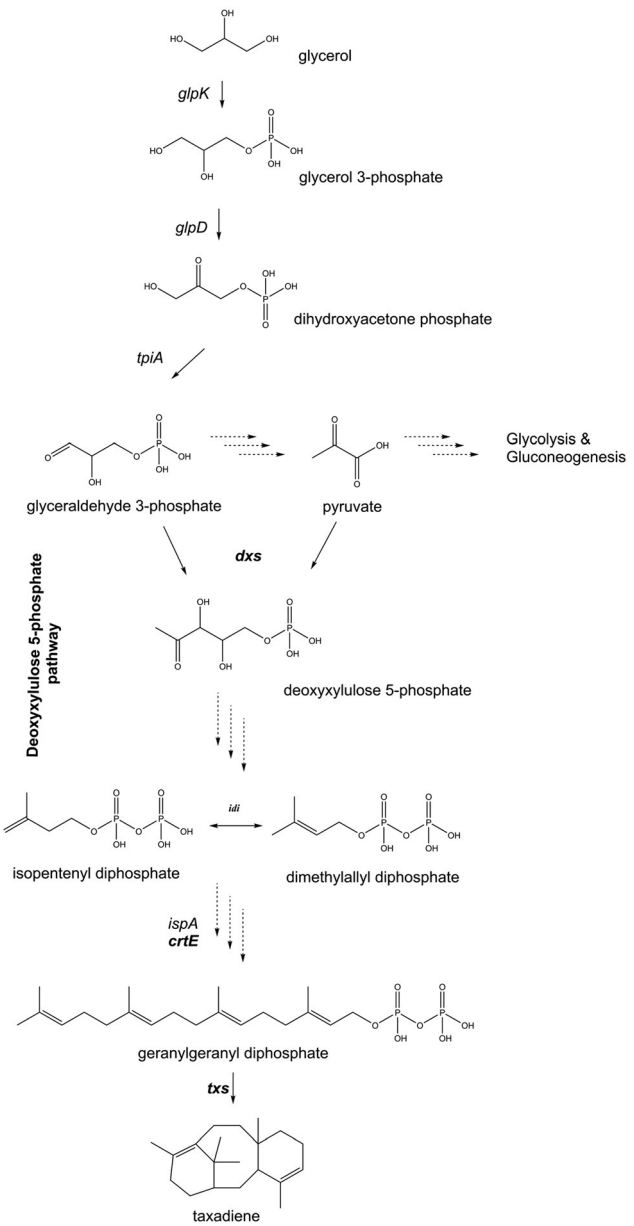

Fig. 1 Biosynthetic pathway from glycerol to TD (overexpressed enzymes are in bold face).

Recent reports have demonstrated that the overexpression of biosynthetic bottleneck enzymes within the deoxyxylulose 5-phosphate pathway (MEP-pathway) provides for significantly improved terpene yields in *E. coli*.[46] In our experimental sets however, the reconstruction of an equivalent inducible synthetic operon (T_7-promotor – induction by isopropyl β-D-1-thio-galactopyranoside) resulted in low TD titers in amounts of 8.77 ± 0.5 mg L^{-1} after 24 h (ESI†). This was traced back to reduced cellular viability after induction, which resulted in a short TD production period. Specifically, *dxs* overexpression potentially led to a marked depletion of the central metabolites, pyruvate and glyceraldehyde 3-phosphate, which are the initial precursors of *E. coli*'s terpene biosynthesis (see Fig. 1).

Therefore, we hypothesized that induction-based terpene production limits the potential of whole-cell bio-catalysis systems. To test this hypothesis, we replaced the T_7-promoter region by lac-I-derived constitutive promoter systems. Such constitutive expression systems do not rely on an inducer molecule, and continuously synthesize the targeted enzymes. The expression rates of the applied constitutive promoters are much lower compared to T_7 promoter systems. Hence, while cells remained in an active growth phase, the metabolic flux from glucose to the desired terpene product remained stable throughout the entire cultivation cycle (see Fig. 2). In addition, switching from an induction-based expression to a constitutive expression system enabled us to efficiently and comparatively screen different synthetic operons for terpene production and productivity (see ESI†). Initially, *txs* was replaced by genes coding for the biosynthesis of the tetraterpene lycopene (*CrtL*, *CrtI*) as both, lycopene as well as TD rely on the diterpene precursor GGDP. Lycopene is a prominent red pigment (*i.e.* tomato and water melon), which is highly suitable for simple visual control of terpene product formation. This system provides for accurate and simple determination of terpene product formation by UV-Vis spectrophotometry and therefore, rapid optimization of heterologous terpene production hosts. Once a heterologous terpene centered operon has been harmonized, with respect to carbon and redox flux balance as well as product yield using lycopene as a read-out system. To implement a metabolically balance host for the desired terpene production, central genetic elements specific to lycopene production, can simply be exchanged with those coding for taxadiene in our study (see ESI†).[47]

Accordingly, our screening approach identified an operon construct combining the genes *dxs*, *idi* and *crtE* that, if co-transformed with the *ctrL–crtI* operon, yielded amounts of 1.26 ± 0.06 mg_{Lyc} g_{cdw}^{-1} lycopene with a growth rate of 0.42 h^{-1} in minimal media. Co-transformation of the *dxs*, *idi* and *crtE* operon with the *txs* coding plasmid yielded significantly improved TD titers with high reproducibility even in shake flask cultures (7.3 ± 0.25 mg_{TD} g_{cdw}^{-1}). By contrast, our initial, inducible T_7-promotor production system yielded only 2.7 ± 0.25 mg_{TD} g_{cdw}^{-1} after 48 h. Consequently, we used our constitutive, whole cell expression system for fed-batch fermentation experiments to efficiently generate TD.

Microbial upcycling and TD production

E. coli is capable of metabolizing a wide range of carbon and nitrogen sources.[48,49] Especially low-value feedstocks, like agro-industrial waste streams, are promising sources for the generation of new sustainable processes.[50,51] However, the utilization of alternative feedstocks, specifically with respect to complex nitrogen sources, can lead to significant productivity modulation.[48] In that regard, we investigated TD production in fed-batch fermentations that were supplemented with either yeast extract (YE) or CSL as main nitrogen source in combination with glycerol as main carbon source. Fermentations using YE under low basal protein expression resulted in a growth rate of μ_{max} = 0.36 ± 0.01 h^{-1} at 30 °C. A TD titer of 274.3 ± 1.0 mg L^{-1} was obtained after 40 h fermentation runtime, and no further changes in TD concentration were detected beyond this time point (see Fig. 3 purple bars). This

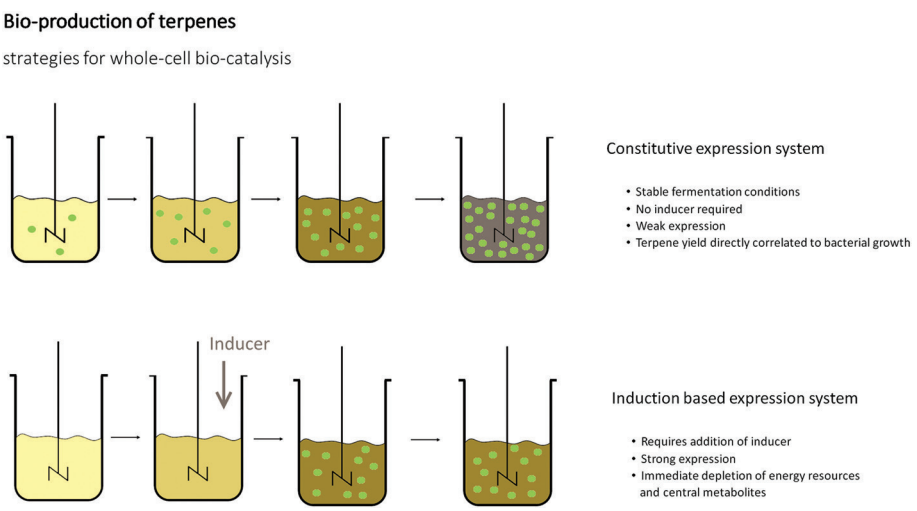

Fig. 2 Constitutive expression and induction-based expression strategy for whole cell biocatalysis of terpenes. Both strategies have been investigated for TD production in this study.

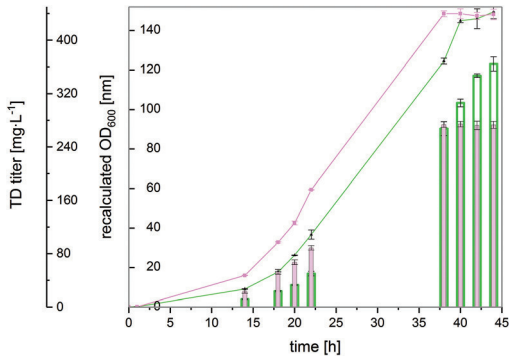

Fig. 3 Time-dependent production of TD in fed-batch fermentations. Fermentation set-ups distinguished between the complex nitrogen sources used (100% YE—purple; mixture of 87.5% CSL and 12.5% YE—green). Lines Represent the calculated OD_{600}; bars represent the TD concentration.

suggests that TD production was directly correlated with bacterial growth. The correlation between cell dry weight (cdw) and TD concentration was calculated and revealed a stable TD amount of 6.65 ± 0.1 $mg_{TD}\ g_{cdw}^{-1}$. This correlation was lower than in our shake flask experiment. We hypothesize that the extraction was not complete or TD was actively depleted by aeration or degradation effects in the reaction medium.

In parallel, we investigated whether CSL, which accumulates as a by-product in the wet corn milling process, is a suitable YE replacement for fed-batch TD fermentation processes. Interestingly, the use of CSL as the main nitrogen source resulted in a 32% (w/v) increase in TD production at the end of the fermentation (see Fig. 3 green bar). Although the maximal growth rate (μ_{max} = 0.18 ± 0.01 h^{-1}) in CSL-based culture was lower than in the YE-based control, a TD concentration of 364.5 ± 10.3 mg L^{-1} was obtained after 44 h. While no active cell growth was observed during the next 3 h, our analyses revealed that the TD concentration increased further during this time. Therefore, we determined the cell dry weight in final samples taken from the fermentation and observed a much lower correlation factor of 0.22 $g_{cdw}\ L^{-1}$ for an OD_{600} of 1 than during the batch fermentation phase (0.37 $g_{cdw}\ L^{-1}$). This effect could be correlated with the high amounts of solid impurities in the fermentation broth derived from the CSL feed solution. In this regard, TD titers were determined in amounts of 9.9 ± 0.6 $mg_{TD}\ g_{cdw}^{-1}$.

Additionally, we investigated the economic gain by replacing main parts of YE from the fermentation process by CSL. Interestingly, the cost per liter cultivation (includes batch and feed solution) could be lowered by more than 35% (see ESI†). Altogether, this highlights the benefit of upcycling CSL. Moreover, this approach demonstrates that further medium and fermentation optimization will lead to higher TD titers.

TD capture by *in situ* dodecane overlay and two-step liquid–liquid extraction

Higher alkanes such as decane or dodecane are often used for diterpene *in situ* extraction in biphasic fermentation systems.[26,30] This diterpene capture process is highly efficient because continuous extraction keeps air-stripping, degradation, and other cultivation-based losses to a minimum.[26,30] However, further processing steps have to be considered and *in situ* extraction strategies have to be evaluated for applicability.

Primarily, the diterpene molecule has to be investigated for its thermal stability, because removing the *in situ* extracting agent is commonly conducted by distillation, which exerts thermal stress on the target compound TD. In parallel, the toxicity and "sustainability" of possible solvents has to agree with the principles of green chemistry. Eventually, in most of the cases a further functionalization step is required to produce biological active diterpenoids. This modification can be carried out *in vivo e.g.* by expressing a cytochrome p450 hydroxylase or *in vitro via* chemically and enzymatically catalyzed reactions respectively. However, if an *in vivo* functionalization step is conducted, premature extraction of the unmodified diterpene molecule drastically lowers titers of functionalized targets when the *in situ* extraction strategy is applied.

Keeping these limitations in mind, we identified dodecane as one promising and suitable *in situ* extraction solvent for diterpenes. TD remained stable under dodecane distillation conditions. Nevertheless, higher alkenes or oils have high boiling points and its or the diterpene molecules separation by distillation would be highly cumbersome and energy intensive. By contrast, lower alkenes like decane, undecane or nonane are toxic and have a negative environmental impact.[52] These extraction reagents can only be applied if they are applied in a minimal fashion or their recovery efficiency exceeds other options. Unfortunately, this cannot be guaranteed in biphasic fermentation systems because solvent loss by aeration or formation of stable emulsions is hardly avoidable. In this regard, we repeated our initial YE and CSL based fermentation process, however, this time using a dodecane overlay of 10% (v/v) fermentation volume, respectively. Interestingly, we did not observe an alteration of the growth rates nor other lasting effects during the fermentation process. This data is in accordance to previous work, which demonstrated that solvents with a log P_{OW} value above 4 do not interact with *E. coli*'s cell membrane (dodecane log P_{OW} = 7).[53]

As dodecane had no effect on the initial fermentation process, we subsequently analyzed TD formation over time in fed-batch fermentation procedures supplemented either with YE or CSL as main nitrogen source. In the presence of CSL the strong formation of stable emulsion (probably dodecane in combination with free peptides) was observed during the fermentation procedure. This effect prevented an accurate determination of the TD concentration (see ESI†). Accordingly, we did not further investigate the process of biphasic *in situ* extraction in combination with CSL as main nitrogen source as almost none of the applied dodecane could be recovered.

In contrast, we were able to demonstrate the benefit of *in situ* extraction by dodecane within the YE based fermentation (see ESI†). More specifically, the TD concentration per cell dry weight (cdw) increased by more than 40% (w/w), affording

9.4 ± 0.5 mg_{TD} g_{cdw}^{-1}. After separating the dodecane phase from the aqueous fermentation broth, we evaporated the dodecane phase to prepare TD for further purification. In this regard, we observed that TD exhibits significant stability (170 °C, <50 mbar, >30 min). After evaporation a very low TD loss of 7% (v/v) was determined. Therefore, we presume that TD is a very stable molecule and that the dodecane mediated TD *in situ* extraction process is a suitable extraction procedure depending on the constituents of the fermentation media. Concerning *in situ* extraction production process the depletion of hydrophobic cultivation media components by the overlay is expected.

Nevertheless, recovery of the dodecane solvent turned out to be challenging. Only 70% (v/v) of the initially applied dodecane could be recaptured (Table 1).

Accordingly, the greenness and sustainability of such a biphasic fermentation process is questionable. Particularly, the spent culture after extraction must be treated as special waste as dodecane evaporates much slower than water under normal conditions. The dodecane loss during the extraction and solvent recovery step contributed over 75% to the total costs of the fermentation process. However, the processing time savings using this *in situ* extraction method lowers personal expenses. Therefore, with regard to economic efficiency the dodecane extraction process must be investigated individually by a thorough techno-economic analysis, which is not focus of this manuscript.

In our alternative TD capture strategy, we devised a convenient procedure based on classical liquid–liquid extraction. The efficiency of liquid–liquid extraction is commonly low, and high product recovery requires repeated and elaborate extraction procedures. As fermentatively generated diterpenes are located in both the supernatant as well as in the cell pellet fractions, the extraction of the entire fermentation broth is required to maximize product yields.[30]

To that end, our high cell-density culture broth required special handling procedures to enable efficient extraction. In order to minimize the effort of extraction, we developed a simple two-step procedure. Initially, the entire cell-containing fermentation broth (1 L) was treated with an admixture of ethyl acetate and ethanol (1 L) that led to cell lysis and liberation of intracellularly accumulated TD. Subsequently, the lysed cell debris were removed by centrifugation. Hexane (500 mL) was added to the resulting supernatant, which induced a phase separation and a reduction of the sample volume (750 mL). This step-wise TD extraction circumvented the formation of a highly stable emulsion as observed with dodecane extraction. In our experience this emulsion is formed only when solvent assisted terpene extraction is conducted in a single step from high cell-density fermentation broths. Notably, our two-step extraction protocol resulted in a 71% (w/v) TD recovery at liter scale. This TD yield could potentially be increased even further by repeated hexane extraction. However, the excessive solvent handling does not justify these extended efforts to maximize TD yield at this point. Moreover, the possibility of fractionated solvent recovery improves the sustainability aspect of classical liquid–liquid extraction. Albeit our liquid–liquid extraction is superior due to the minimization of emulsion formation. Furthermore, it is also suitable for thermally labile or functionalized terpenes.

Table 1 Evaluation of both strategies investigated in this study for TD extraction from 1 L fermentation broth (*in situ* product removal, two step liquid–liquid extraction)

		Extraction strategies 1 L TD fermentation broth		
		In situ (YE)	Liquid–liquid (YE)	Liquid–liquid (CSL)
Solvents [L] and Recovery [%]	Dodecane	0.1 70	—	—
	Hexane	—	0.5 90	0.5 90
	Ethyl acetate		0.5 n.d.	0.5 n.d.
	Ethanol	—	0.5 n.d.	0.5 n.d.
Evaporation	Solvent volume [L]	0.07	0.75	0.75
	Energy required [kJ]	~16	~200	~200
	TD recovery [%]	93	99	99
Results	Expenses solvent	+++	+	+
	Special waste	+++	+	+
	Time	+	++	++
	Produced TD [mg]	~394	~274	~364
	Extracted TD [mg]	~256	~195	~260

TD purification by CPC

For further TD purification, the extracts obtained, whether by *in situ* dodecane overlay or by post-cultivation liquid–liquid extraction, were evaporated until an oily mixture that predominantly consisted of lipids with a TD content below 10% (w/v) remained. The initial purification strategy involved an isocratic (100% acetonitrile (w/v)) reversed phase HPLC (RP-HPLC) purification method where the oily mixture was mixed with acetonitrile. However, this re-solubilization approach resulted in the formation of immiscible oil drops/resins that incorporated a substantial amount of TD. Only significant dilution with more acetonitrile led to complete dissolution of the mixture. Thus, many feed injections would have been necessary to completely purify the produced TD. The screening of other solvent systems that are classically used in RP-HPLC protocols did not solve this admixing issue. Therefore, we set out to develop an alternative downstream technology, due to this universal issue concerning diterpene purification from microbial-derived extracts. Eventually, we devised a new CPC-based purification protocol inspired by sesquiterpenoid purification from plant material.[54] This liquid–liquid chromatographic separation technology uses two immiscible liquid phases, one serving as a mobile and the other as a stationary phase. The mobile phase is pumped through the stationary phase, whereas the latter is kept in the column by applying a centrifugal force field. The separation of the applied sample is achieved by differential partitioning of the mixture compounds between

the two phases. In order to achieve sufficient separation in terms of resolution, productivity and solvent consumption, a biphasic system in which the partition coefficient (K) of the target compound is in the range of $0.5 < K < 2.5$ is preferred.[55] Initially, various biphasic solvent systems were screened. The predictive thermodynamic model "Conductor-like Screening Model for Realistic Solvation" was used for the pre-selection of a suitable solvent system in order to reduce experimental effort as described by Hopmann *et al.*[56,57] On the basis of the molecular structure of TD, *K*-values were predicted in commonly used CPC solvent systems, including hexane/ethyl acetate/methanol/water, heptane/ethyl acetate/methanol/water, butanol/methanol/water, hexane/ethyl acetate/acetonitrile, and hexane/ethanol/acetonitrile. Various compositions of these systems were screened in order to cover a wide polarity range. In that regard, a hexane/ethanol/acetonitrile biphasic system was computationally selected as a prime target for TD purification. Subsequently, an experimental evaluation using shaker flask experiments was performed and the respective TD *K*-values determined for different solvent compositions. An *n*-hexane/ethanol/acetonitrile system with proportions of 4.33/1.0/1.07 (v/v/v) was ultimately selected and applied for TD separation by CPC. The stationary phase retention (SF) determined for this system was 0.56 at a mobile phase flow rate of 8 mL min^{-1}. After sample injection, constant stationary phase loss (2 mL min^{-1}) was detected over the entire runtime. The collected fractions were analyzed by GC-MS. TD eluted between minute 43 and 48, and TD purity and recoveries were determined by GC-flame ionization detection (FID). The final purification process produced TD that was 95% pure with a recovery of 95% (Fig. 4).

This purification process allowed for rapid and efficient isolation of the sample diterpene TD. Specifically, sample dissolution with the lower phase of the biphasic system did not lead to the incorporation of the diterpene molecule into immiscible oil droplets. This significantly raised the process efficiency because of the increased TD mass load in CPC purification compared with our initial RP-HPLC separation process. Additionally, although irreversible adsorption of the diterpene macrocycle scaffold to solid phases is particularly responsible for losses in RP-HPLC diterpene purification, this does not apply to CPC, resulting in improved recovery. Therefore, we recommend CPC as an alternative to the classically used RP-HPLC purification processes for diterpene purification from high cell density fermentation broth.[22]

Lipase-mediated epoxidation of TD

Most of the terpenoids mentioned in the dictionary of natural products harbor at least two oxygen-containing functional groups.[37] These groups, in conjunction with the high stereo complexity and density of the carbon atoms within diterpenoids, commonly convey biological functions.[18,35,36] Notably, natural diterpene epoxides such as dictyoepoxide, dollabellanone, and calyculone have been found to act as a vasopressin receptor agonist, an anti-protozoal agent, and a cytotoxic agent, respectively.[58–60] Moreover, epoxides can be chemically diversified further into ketones, alcohols (diols, thiols, amino alcohols, *etc.*), and polymers, thereby expanding the chemical space.[61–64] To date, epoxidation of monoterpenes has been mainly investigated because of their abundant availability in plant extracts.[65–69] However, Dolabellatriene, a natural diterpene found in algae and corals, was also recently reported to be a suitable target for a lipase-mediated epoxidation reaction.[70] In contrast to chemical epoxidation protocols, enzymatically mediated reactions are highly specific and can be conducted under milder conditions.[68,69,71]

In this study, we investigated a lipase (*CalB*)-mediated TD epoxidation reaction. In order to slowly release H_2O_2 into the reaction medium, we used environmentally compatible urea-hydrogen peroxide.[68] As depicted in Fig. 5, acetic acid primarily reacts with hydrogen peroxide to form a per-oxo acid. This per-oxo-acetic acid targets the olefinic bonds of TD either from the *re*- or *si* face, which theoretically would results in 4 stereo-isomeres. The epoxidation reaction was monitored by time-resolved GC-FID analysis of samples that were regularly drawn from the reaction vessel (0.75 h intervals). Detailed

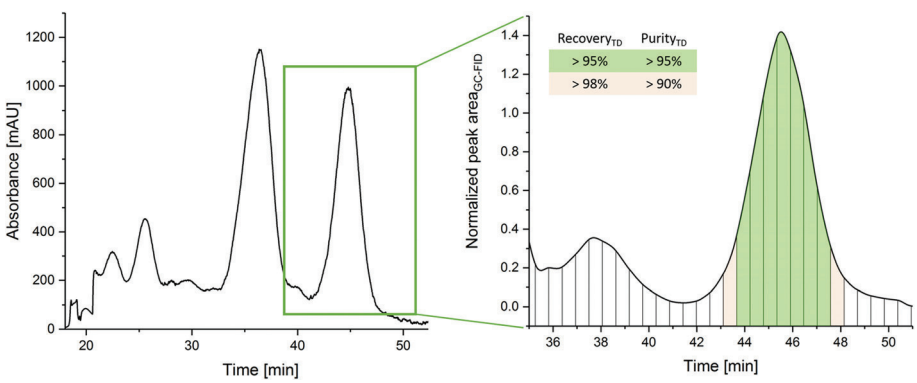

Fig. 4 Left: On-line chromatogram at 210 nm of a centrifugal partition chromatography batch seperation process of the TD-containing crude extract that was obtained from *E. coli* fed-batch fermentation. The solvent system of *n*-hexane/acetonitrile/ethanol/4.33/1.0/1.07 (v/v/v) was applied (descending mode: lower phase as mobile phase, 8 mL min^{-1} mobile phase flow rate, 1700 rpm, cinj TD ≈ 16 mg mL^{-1}, vinj = 2 mL). Right: Off-line chromatogram of the fractions in close proximity to the TD retention time reconstructed by GC-FID analyses.

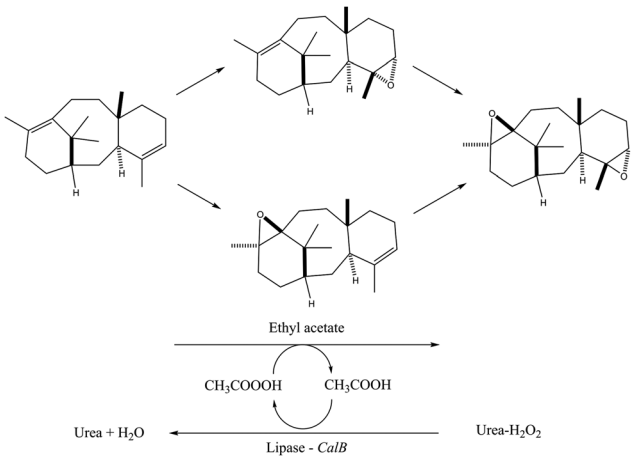

Fig. 5 Reaction mechanism from TD to bis-epoxilated taxanoids by lipase (*CalB*) mediated epoxidation reaction.

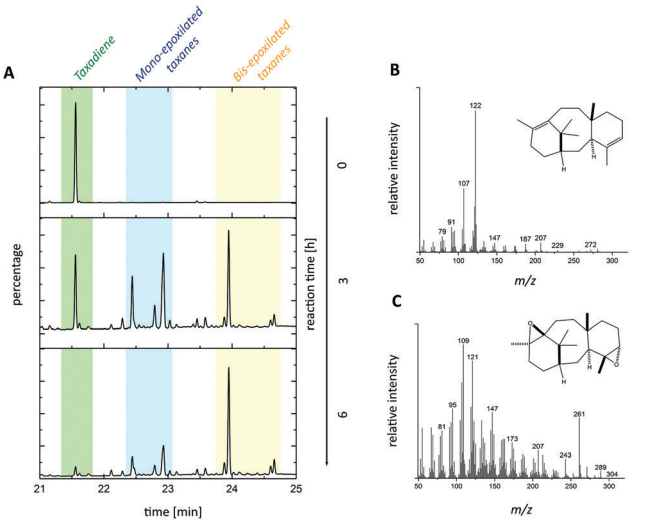

Fig. 6 A: Time-resolved epoxidation reaction from TD (green), over mono-epoxilated taxanoids (blue) to bis-epoxilated taxanoids (yellow). B: MS-spectra of TD. C: MS-spectra of the major product taxa-4(5),11(12)-bisepoxide.

GC-MS analyses of the sample revealed the formation of mono-epoxides (*m*/*z* 288) prior to a second epoxidation step that generated bis-epoxilated taxanoid compounds (*m*/*z* 304) (see Fig. 6).

Interestingly, we noticed a predominant formation of a single bis-epoxid species that was isolated by thin layer chromatography and subsequently structurally characterized. The 1D and 2D-NMR spectroscopy data of this compound were consistent with reported chemical shifts of taxa-4(5),11(12)-bisepoxide (ESI†).[71]

These GC-MS and NMR datasets indicate that the complex stereochemistry of TD and this reaction set-up resulted in the generation of taxa-4(5),11(12)-bisepoxide as the major product. Subsequently, we fine-tuned the reaction conditions towards

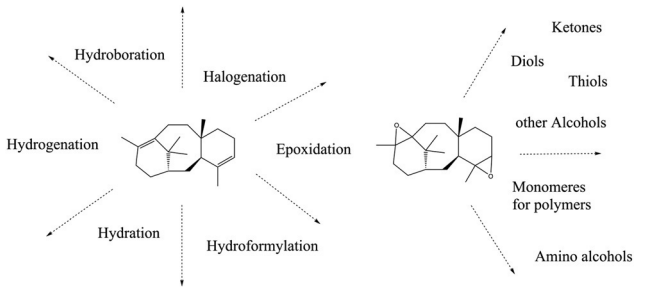

Fig. 7 Possible routes for expanding the taxanoid chemical space based on our holistic process for TD and taxa-4(5),11(12)-bisepoxide molecules production.

the exclusive production of this molecule. The enantiomeric excess was increased from an initial 58.3% to 83.1% simply by lowering the reaction temperature from 30 °C to 20 °C (ESI†). Under these conditions, TD conversion was complete after 6 h and *in situ* product stability was monitored for a period of another 2 h. TD oxy-functionalization outside the cellular environment can circumvent common issues that are inherent to the *in vivo* targeted taxanoid production. In that regard, intracellular hydroxylation conventionally leads to a dramatic decrease in taxanoid titers due to low enzyme transformation rates and to decreased viability from the enhanced metabolic burden.[27] Moreover, the heterologous expression of the native cytochrome p450 5-α-TD hydroxylase leads to a diverse range of products, which complicates the efficient production of single taxanoids in whole-cell biocatalysis approaches and reduces the yield of target compounds.[72]

In addition, the epoxides generated in this study are highly reactive, and their chemical space can be easily diversified in order to obtain new taxanoid structures with potential pharmacological activities (see Fig. 7).

Conclusions

This study describes a fast and sustainable fermentative production of the structurally complex natural compound TD and its subsequent chemo-enzymatic functionalization. In order to provide practical access to taxanoid molecules, initially different TD extraction methods from *E. coli* high cell-density cultures were evaluated and optimized with a particular focus on further recovery and purification options. Subsequently, a new purification process for diterpenes from biotechnological fermentation broth using CPC was developed that resulted in high TD recovery and purity. The obtained TD was further functionalized *in vitro* by a lipase mediated epoxidation reaction. Most notably, this epoxy-functionalization approach resulted in the highly specific generation of taxa-4(5),11(12)-bisepoxide that can be easily converted to a diverse array of taxanoids using standard chemical reactions (see Fig. 7). This situation contrasts with the commonly applied *in vivo* cytochrome P450 hydroxylation approaches that usually exhibit low reaction rates and the generation of multiple products.

All the process steps involved in TD generation, purification and functionalization can be transferred to commercially relevant scales, with a particular focus on economic and ecological constraints. For the first time, the processes reported here facilitate the ready availability of the TD molecule and its functionalized congeners. The technology platform described in this study enables a biology-oriented synthesis of *ad hoc* designed bioactive taxanoids.

Experimental

Materials

Fermentation media components were obtained at highest purity from Roth chemicals and Applichem GmbH. CSL was purchased from Sigma-Aldrich. Extraction was performed with technical grade ethanol, ethyl acetate, and hexane from Westfalen AG. For all other procedures, highest purity grade chemicals were used. In this context, acetic acid, acetonitrile, chloroform, ethyl acetate, and hexane were obtained from Roth chemicals. Immobilized Lipase B from *Candida Antarctica* (*CalB*), $CDCl_3$, isopropyl-β-D-thiogalactopyranoside and urea-hydrogen peroxide were purchased from Sigma-Aldrich.

Bacterial strains and growth conditions

All strains used were obtained from Merk Millipore. For cloning, *E. coli* HMS 174 (DE3) was used and grown at 37 °C in Luria–Bertani medium. The screening of the constructed lycopene production platform library is described in the ESI.†

E. coli BL21 (DE3) grown in R-Media[73] supplemented with 10 g L^{-1} glycerol instead of sucrose and 12 g L^{-1} YE or 10.5 g L^{-1} CSL in combination with 1.5 g L^{-1} YE was used for TD production at 30 °C (shake flask experiments and fermentation batch medium). Induction of T_7-promoted TD production was initiated at OD_{600} = 0.2 by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. Chloramphenicol (34 μg mL^{-1}) and Kanamycin (50 μg mL^{-1}) were added as required.

Cloning

Genes coding for deoxyxylulose 5 phosphate synthase (*dxs*; GenBank: YP001461602.1), isopentenyl-diphosphate delta isomerase (*idi*; GenBank: AAC32208.1), geranylgeranyl diphosphate synthase (*crtE*; GenBank: KPA04564.1), taxadiene synthase (txs; GenBank: AAK83566.1), phytoene desaturase (CrtB; GenBank: AHG94990.1) and Phytoene synthase (CrtL; GenBank: AHG94989.1) were used.[74] The construction of polycistronic operons were conducted by standard BioBrick cloning and adjusted as described previously.[75,76] *Dxs* was amplified from original sources by PCR. The other genes were synthesized by Life Technologies GmbH, and the codon usage was optimized for *E. coli*. Primers were obtained by Eurofins Genomics, and plasmids were obtained from Novagen/Merk Millipore. The Operons were set under constitutive expression by lac-I-derived promoters.[77]

Analytics and TD quantification

Analysis of TD was performed using a Trace GC-MS Ultra with DSQII (Thermo Scientific). One microliter (1/10 split) of the sample was loaded using a TriPlus auto sampler onto an SGE BPX5 column (30 m, I.D 0.25 mm, film 0.25 μm). The injection temperature was 280 °C, and helium was used as the carrier gas. Initial column temperature was set to 50 °C and maintained for 2.5 min before a temperature gradient at 10 °C min^{-1} up to 320 °C was applied. The final temperature was held for an additional 3 min. MS data were recorded at 70 eV (EI) as total ion current, and *m*/*z* was given as relative intensity (%). The recorded *m*/*z* range was between 50 and 650. The same GC protocol was followed for FID.

NMR spectra were recorded in $CDCl_3$ with an Avance-III 500 MHz (Bruker) at 300 K. ^1H NMR chemical shifts are given in ppm relative to $CDCl_3$ (δ = 7.26 ppm). ^{13}C NMR chemical shifts are given in ppm relative to $CDCl_3$ at δ = 77.16. The 2D experiments (HSQC, HMBC, COSY, and NOESY) were performed using standard Bruker pulse sequences and parameters.

For TD quantification, the purified compound was evaporated to dryness, weighed and dissolved at different concentration in hexane before analyzed by GC-FID. A standard curve of TD concentration over the respective GC-FID peak area was produced, and a correlation factor was calculated (ESI†).

Fermentation

Fermentation was performed in a DASGIP® 1.3 L parallel reactor system (Eppendorf AG). An overnight pre-culture was used for the inoculation of fermenters (OD = 0.1) that differed in the supplemented nitrogen source. The cultivation temperature was kept constant at 30 °C. Initial stirring velocity and airflow was set to 200 rpm and to 0.2 volumes of air per volume of medium per min (vvm), respectively. Dissolved oxygen was kept at 30% by successive increases of the stirrer velocity, the oxygen proportion, and eventually the airflow. A pH value of 7.00 was controlled by the addition of 25% aqueous ammonia. A pH value shift above 7.05 initiated a feed shot of 40 mL. The feed solution consisted of 600 g L^{-1} glycerol, 20 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 mg L^{-1} thiamine–HCl, 16 mL 100× trace elements solution (5 g L^{-1} EDTA; 0.83 g L^{-1} $FeCl_3$-$6 \cdot H_2O$; 84 mg L^{-1} $ZnCl_2$, 13 mg L^{-1} $CuCl_2$-$2 \cdot H_2O$, 10 mg L^{-1} $CoCl_2$-$2 \cdot H_2O$, 10 mg L^{-1} H_3BO_3, and 1.6 mg L^{-1} $MnCl_2$-$4 \cdot H_2O$), and 40 g L^{-1} complex nitrogen source (pH = 7.00). Complex nitrogen sources besides 100% YE consisted of 87.5% CSL mixed with 12.5% YE. Samples were taken at different time points to determine the OD_{600} and the TD content.

TD extraction and its preparation for the purification process

In situ extraction was conducted by the addition of 10% (v/v) dodecane after 25 h of cultivation time. TD quantification was conducted by analyzing the TD content in the dodecane phase. Therefore, samples taken were centrifuged for 2 min at 10 000 rpm and 100 μL of the upper phase taken, diluted 1 : 10 with hexane and analyzed by GC-FID.

After the fermentation was terminated the whole fermentation broth was centrifuged for 20 min at 10 000 rpm. The dodecane phase was separated by a separation funnel. The recovered dodecane was completely evaporated prior to re-solubilization.

For the other extraction strategy investigated, samples were regularly taken from the fermenter in the range from 0.5 to 10 mL, depending on the OD_{600}, and diluted as required to a final volume of 10 mL. Thirty milliliters of a solvent mixture that consisted of ethanol, ethyl acetate, and hexane (1 : 1 : 1) (v/v/v) was added and vigorously mixed for 1 h at 30 °C. The solution was centrifuged for 2 min at 10 000 rpm. Subsequently, the upper organic phase was directly analyzed by GC-FID for TD quantification.

For the extraction of the whole cultivation broth, equal amounts of ethyl acetate and ethanol were added to the cell culture in a ratio of 1 : 1. This first extraction step was performed for 12 h at 30 °C on a rotary shaker (60 rpm). Subsequently, the extract was centrifuged for 15 min at 7000 rpm, the supernatant separated from the pelleted cell debris, and hexane added (25% of the supernatant volume). The mixture was shaken for an additional 3 h before the phases were separated using a separation funnel. The upper phase was evaporated until an oily resin remained.

For the re-solubilization and preparation for CPC purification of the oily resin, a biphasic system consisting of *n*-hexane/ethanol/acetonitrile 4.33/1.0/1.07 (v/v/v) was prepared and separated by a separation funnel. 20 mL of the lower phase was mixed with the TD enriched oil (approximately 5 mL) and subsequently centrifuged for 5 min at 12 000 rpm. The soluble fraction was applied for CPC purification.

Purification of TD

Purification was performed at room temperature in a SCPC 250 unit from Armen Instrument (now called CPC 250, Gilson Purification SAS). Two preparative HPLC pumps (maximal flow rates of 50 mL min^{-1}) were used for filling the column with stationary phase and for pumping the mobile phase during the separation process, respectively. The CPC column volume was 182 mL. The feed sample was introduced through a six port manual injection valve and a 2 mL injection loop was used. The effluent was monitored with a UV-Detector (ECOM DAD600 2WL 200–600 nm) at 210 nm.

The biphasic liquid system, *n*-hexane/ethanol/acetonitrile 4.33/1.0/1.07 (v/v/v), was prepared at room temperature (22 °C) by mixing the corresponding volume portions of the solvents. The mixture was vigorously shaken and equilibrated for at least 2 h before being split with a separation funnel.

The CPC column was filled with the upper (stationary) phase, and the instrument's rotational speed was set at 1700 rpm. The lower phase (mobile) phase was pumped with a flow rate of 8 mL min^{-1} until no more stationary phase eluted from the column, *i.e.* hydrodynamic equilibrium was achieved. Subsequently, CPC separation was initiated by an injection of 2 mL of sample while a 1700 rpm rotational speed and a mobile phase flow rate of 8 mL min^{-1} in descending mode were applied. Fractions were collected during the separation process using a fraction collector (LS 5600, Armen Instrument) and analyzed by GC-FID.

Lipase-mediated epoxidation of TD

For the epoxidation reaction 1 mg mL^{-1} TD, 0.1 mg mL^{-1} acetic acid, and 2 mg mL^{-1} immobilized *CalB* were added to 5 mL ethyl acetate. In order to keep temperature constant at 20 °C, 25 °C, or 30 °C, the reaction vessel was placed in a thermoblock (Eppendorf AG). Reactions were initiated by the addition of 2 mg mL^{-1} urea-hydrogen peroxide, which was dissolved in ethanol as a stock solution of 100 mg mL^{-1}, before shaking at 1000 rpm. Samples were taken after different time points, and the reaction monitored over time by GC-MS and GC-FID analysis.

Conflicts of interest

The authors declare no conflict of interests.

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5 IDENTIFICATION OF SESQUITERPENE SYNTHASES FROM THE BASIDIOMYCOTA *CONIOPHORA PUTEANA* FOR THE EFFICIENT AND HIGHLY SELECTIVE β -COPAENE AND CUBEBOL PRODUCTION IN *E. COLI*

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5.1. AUTHORS' CONTRIBUTIONS

WOLFGANG MISCHKO: Conceiving the project and bioinformatic based selection of suitable uncharacterized TPSs candidates. Planning and execution of experiments; in particular heterologous expression of the novel TPSs in optimized *E. coli* production strains as well as process development for yield-optimized terpene generation. Furthermore, fermentation and product purification in reactor scale as wells as enzyme screening, characterization, compound evaluation and structure elucidation. Moreover, comprehensive data analysis, amino acid sequence based phylogenetic analysis as well as supervision of students and preparing the entire manuscript.

MAX HIRTE: Comprehensive support, especially in conceiving the project, protein modelling and analysis.

MONIKA FUCHS: Supervision of the research project

NORBERT MEHLMER: Supervision of the research project

THOMAS B. BRÜCK: Project lead and cooperation in writing the manuscript as well as its critical revision.

5.2. SUMMARY

Recent data of whole genome projects concerning various mushroom-forming fungi (Basidiomycota) species represent a largely unexplored source of biotechnological potential. Only a very small part of the fungal terpenome has so far been investigated and characterized due to a lack of experimental studies. The article "Identification of sesquiterpene synthases from the Basidiomycota *Coniophora puteana* for the efficient and highly selective β-copaene and cubebol production in *E. coli*" focuses on the discovery, investigation and industrial exploitation of new STPs from *C. puteana* (brown-rot fungus). Based on bioinformatic sequence analysis and conserved motif assessment, three annotated but up to that time completely uncharacterized TPSs (Copu1 - Copu3) were selected. The corresponding gene sequences were cloned into constitutive expression systems for enzyme activity- and product range screenings. The heterologous expression in metabolically engineered *E. coli* led to the identification of two previously unknown STPs. Terpene production and characterization was evaluated by GC-MS spectrometry and NMR spectroscopy. The discovered synthases exhibit the highest reported product selectivity for their main compounds and represent the first

exclusive enzymes for β-copaene (62% product selectivity) and cubebol (75% product selectivity) generation.

While cubebol is of commercial interest concerning its strong refreshing effect as food and flavor additive, little is known about β-copaene and its detailed biological function. However, a complete chemical conversion of β-copaene to the better-characterized α-isomer was achieved. This compound is associated with invaluable biological activities, including potential anticarcinogenic and neuroprotective applications.

An efficient fermentation process was developed utilizing collagen hydrolysate and glycerol, which represent both low value and renewable raw materials. In combination with a metabolically optimized heterologous production system, microbial cell factories for the de novo production of 215 mg/L β-copaene and 497 mg/L cubebol were created. The achieved terpene titers exceeded all published biotechnological data at this time and offer a promising economic alternative to extraction from natural plant sources or total chemical synthesis. A comprehensive phylogenetic analysis was carried out providing insight into the fungal terpenome as well as the structural and functional diversity of STPs from Basidiomycota. The results of amino acid sequence analysis revealed a correlation between synthase sequence similarity and corresponding product profile. This highlights the potential for *in silico* prediction of unknown TPSs and their functionality.

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RESEARCH

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Identification of sesquiterpene synthases from the Basidiomycota *Coniophora puteana* for the efficient and highly selective β-copaene and cubebol production in *E. coli*

Wolfgang Mischko, Max Hirte, Monika Fuchs, Norbert Mehlmer and Thomas B. Brück*

Abstract

Background: Terpenes are an important and extremely versatile class of secondary metabolites that are commercially used in the pharmaceutical, food and cosmetics sectors. Genome mining of different fungal collections has revealed the genetic basis for a steadily increasing number of putative terpene synthases without any detailed knowledge about their biochemical properties. The analysis and research of this rich genetic source provides a precious basis for the advancing biotechnological production of an almost endless number of valuable natural metabolites.

Results: Three annotated terpene synthases from the little investigated Basidiomycota *Coniophora puteana* were studied in this work. For biochemical characterization, the heterologous expression in *E. coli* was conducted leading to the identification of two sesquiterpene synthases capable of the highly selective generation of β-copaene and cubebol. These compounds are commercially used as food and flavor additives. The new enzymes show the highest reported product selectivity for their main compounds and therefore represent the first exclusive synthases for β-copaene (62% product selectivity) and cubebol (75% product selectivity) generation. In combination with an optimized heterologous microbial production system, we obtained product titers of 215 mg/L β-copaene and 497 mg/L cubebol.

Conclusion: The reported product selectivity and our generated terpene titers exceed all published biotechnological data regarding the production of β-copaene and cubebol. This represents a promising and economic alternative to extraction from natural plant sources and the associated complex product purification.

Keywords: Copaene, Cubebol, Sesquiterpene, Basidiomycota, *Coniophora puteana*, Heterologous expression, Fermentation, Phylogenetic analysis

Background

Filamentous fungi are experts at producing highly complex natural compounds of commercial interest [1]. Fungal-derived polyketides have been the main focus of recent research activities, whereas the identification of terpenoids and their biosynthesis in fungi have received little attention although these compounds represent the most structurally diverse group of natural products [2]. All terpenoids are based on the same basic C_5 isoprene building blocks, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which are consecutively fused by head to tail condensation. Depending on their carbon chain length, these linear phosphorylated alkenes are universal precursors of mono(C_{10})-, sesqui(C_{15})-, di(C_{20})-, sester(C_{25})- or tri(C_{30})-terpenes [3]. The structural diversity within the class of terpenoids results from the complex cyclization of the linear precursors into chemically complex molecules, a reaction catalyzed by the family of terpene synthase (TPS) enzymes. More specifically, sesquiterpene

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synthases (STPSs) transform the linear C_{15} precursor farnesyl pyrophosphate (FPP) into a variety of different scaffolds, which form the structural core of functionalized, bioactive sesquiterpenoids (STPs) [2]. Many STPs are lead structures in pharmaceutical applications, encompassing anti-cancer [4, 5], anti-inflammatory [6, 7] and antibiotic [8] therapies. STPs also have existing applications in the food and cosmetics industries, where they are used as flavor and fragrance ingredients [9, 10].

As the extraction of these latter compounds from natural sources is often cost-intensive and not suitable to meet market demands [11], effective biotechnological production routes are the focus of development efforts [12]. Due to the rapid progress of modern sequencing techniques, in silico genome mining based on conserved amino acid motifs can be applied to identify putative TPSs [13]. Whole genome projects of different mushroom-forming fungi (Basidiomycota) species represent a largely unexplored source of investigation and extraction of rarely characterized STPSs [14, 15]. In this context, numerous putative TPSs have already been annotated but their catalytic capacities remain to be established [14]. To exploit the biotechnological potential of fungal biosynthetic pathways, subsequent functional expression and characterization of these enzymes is required. This represents the first step in providing a sustainable supply of high-value natural products using microorganisms as cell factories [16].

Based on the available genome data, we were able to select potential TPSs from the Basidiomycota *Coniophora puteana*, which is classified as a common wood rotting fungus [17]. At present, only the enzyme systems involved in *C. puteana*-dependent wood depolymerization have been characterized in detail [18, 19]. However, there are no reports of any other enzymes involved in secondary metabolite production. Therefore, this study focuses on the identification and characterization of three putative *C. puteana*-derived TPSs. We present the functional reconstitution of these enzymes in an *E. coli* whole-cell production system. With respect to designing an effective STP production system, the supply of the FPP precursor needed to be ensured by an adapted co-expression of bottleneck enzymes (DXS, Idi) from the native non-mevalonate pathway (MEP) (Fig. 2a). This optimization measure ensured a directed carbon flux towards STP production. Two of the three identified TPSs from *C. puteana* (Copu1-3) could be expressed functionally in *E. coli*, resulting in a range of sesquiterpene products. The main product of the Copu2 fermentations was the tricyclic β-copaene. By contrast, Copu3 fermentations provided cubebol as the main product, which is approved as a dietary supplement and flavoring agent [9, 20] due to its pronounced cooling effect [21]. Utilizing the new terpene synthases from *C. puteana* within an optimized production construct provided 215 mg/L β-copaene and 497 mg/L cubebol. The cubebol production titers reported in this study exceeded all other described production systems by a factor of 50.

Results

Identification and characterization of putative terpene synthase genes in C. *puteana*

A Basic Local Alignment Search Tool (BLAST) analysis of fungal genomes with conserved terpene synthases sequences resulted in the identification of a large number of putative terpene synthases (TPSs). However, for the majority of TPS candidates, a biochemical and functional characterization remains to be established. In order to gain insight into their catalytic function, three putative TPSs (Copu1: XP_007772164.1; Copu2: XP_007771895.1; Copu3: XP_007765978.1) from *C. puteana* were selected for cloning and functional characterization. The specific selection was made on the basis of characteristic conserved sequence motifs. Moreover, Copu1-3 showed closely related amino acid (AA) sequences (55–62% similarity). A comparison of the AA sequence of Copu1 and Copu2 with the public database showed < 50% similarity to other listed enzymes, covering all biological realms. By contrast, Copu3 showed 65% similarity to putative TPS sequences, which were not functionally characterized. The AA sequences of all three enzymes contained typical sequence motifs common to the TPS family, such as the highly conserved (N/D)D(L/I/V)x(S/T)xxxE (NSE) triad and the aspartate-rich D(D/E)xxD motif, coordinating a trinuclear Mg^{2+} cluster, which is catalytically essential for the initial hydrolysis of the FPP-derived pyrophosphate group [22] (see Additional file 1). A highly conserved arginine residue, indicated as the pyrophosphate sensor, is located 46 positions upstream of the NSE triad. Additionally, the catalytically important RY-dimer, which is involved in the formation of hydrogen bonds to the substrate-derived pyrophosphate, is found 80 AA downstream of the NSE triad and close to the C-terminus [23–25].

Heterologous expression of *C. puteana* TPS genes resulted in the generation of diverse sesquiterpenes in *E. coli*

To study the product profile of Copu1-3, their predicted coding sequences were codon-optimized and synthesized for transfer into *E. coli* expression vectors. For the heterologous expression, an adapted production system based on a single operon with a constitutive promoter was constructed. Reported bottleneck enzymes from *E. coli*'s native non-mevalonate pathway (MEP) were selected for co-expression (DXS; WP_099145004.1 and idi; AAC32208.1) to increase

the precursor supply and enhance the general isoprenoid production (Fig. 1). The resulting plasmids were transformed into *E. coli* HMS174 (DE3) for recombinant gene expression and subsequent analysis of new, potential terpene compounds. In the first series of experiments, a geranylgeranyl diphosphate synthase (*Pantoea ananatis*; crtE; ADD79325.1) was additionally integrated into the operon to study a potential function as diterpene synthases. *E. coli* cultures co-expressing Copu2 or Copu3 with the MEP bottleneck enzymes for 48 h produced a mixture of exclusively 12 and 18 terpene products, respectively. A cultivation temperature of 30 °C and reduced shaking (90 rpm) ensured an adequate bacterial growth rate and the requirements for the production of potentially volatile compounds. Gas chromatography–mass spectrometry (GC–MS) analysis of the liquid extract revealed new product peaks in both cultures with typical mass fragmentation patterns at 161, 207, 222 m/z and 105, 161, 204 m/z (Fig. 2), illustrating the mass patterns for cyclic C_{15} hydrocarbons with and without a single hydroxyl group. Copu3 appeared to be quite selective for the generation of one major STP alcohol (RT: 15.4 min; parent ion at 222 m/z). In contrast, Copu2 appeared to convert FPP into a smaller and slightly different set of cyclization products. The major product of Copu2 fermentations (RT: 14.3 min) was identified as an unhydroxylated STP compound (parent ion at 204 m/z). By contrast, no terpenoid products were detected in *E. coli* cultures expressing Copu1. Neither the co-expression with a GGPP synthase to enable a possible formation of diterpenes nor the evaluation of different fermentation temperatures to avoid eventual evaporation of volatile compounds (e.g., monoterpenes) showed any product formation. Therefore, Copu1 was classified as a non-functional TPS sequence.

Terpene purification and structure elucidation

A putative identification of the newly generated STPs was performed by comparing their detailed mass spectra to the National Institute of Standards and Technology (NIST) Database. The comparison of the GC–MS metabolite profiles of *E. coli* cultures expressing Copu2 revealed the putative tricyclic STP β-copaene (RT: 14.3 min; parent ion at 204 m/z; major daughter ions at 105, 161 m/z) as the major product. β-Copaene accounted for about 62% of the total STPs detected by GC-FID. *E. coli* strains expressing Copu3 accumulated the putative tricyclic STP alcohol cubebol (RT: 15.4 min; parent ion at 222 m/z; major daughter ions at 105, 161, 207 m/z) as the major product, representing 75% of the total terpene fraction (Fig. 2). Other minor compounds from both cultures encompassed δ-cadinene, β-elemene, germacrene D and germacrene D-4-ol, whose GC–MS spectra were consistent with NIST database references. Notably, several minor STPs did not match any NIST reference spectra and could therefore not be assigned. In addition to GC–MS analyses, the main products of Copu2 and Copu3 were analyzed by NMR spectroscopy. Organic extraction allowed HPLC-based β-copaene and cubebol purification from 1-L fermentations. The HPLC separation of the Copu2 fermentation products allowed for the extraction of the main product but could not resolve several other STPs, which most likely represent a mixture of different product isoforms. However, another minor product was isolated from the Copu3 fermentation broth.

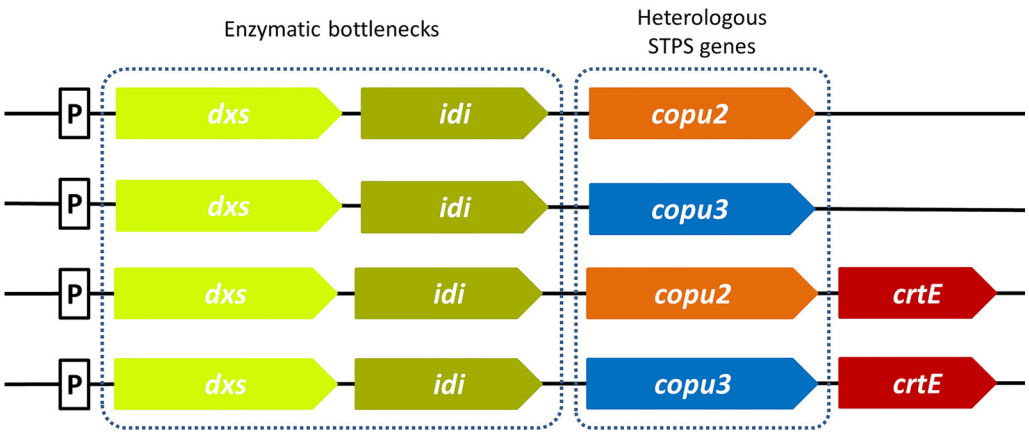

Fig. 1 Design of the synthetic operons for an increased precursor supply and efficient STP production. Enzymatic bottlenecks within the upstream non-mevalonate (MEP) pathway (dxs and idi) as well as the heterologous STPS genes (Copu2 and Copu3) are combined in a single synthetic operon. The integration of an additional geranylgeranyl diphosphate synthase (crtE) allowed to study a potential function as diterpene synthases. A constitutive promoter (P) regulates the pathways

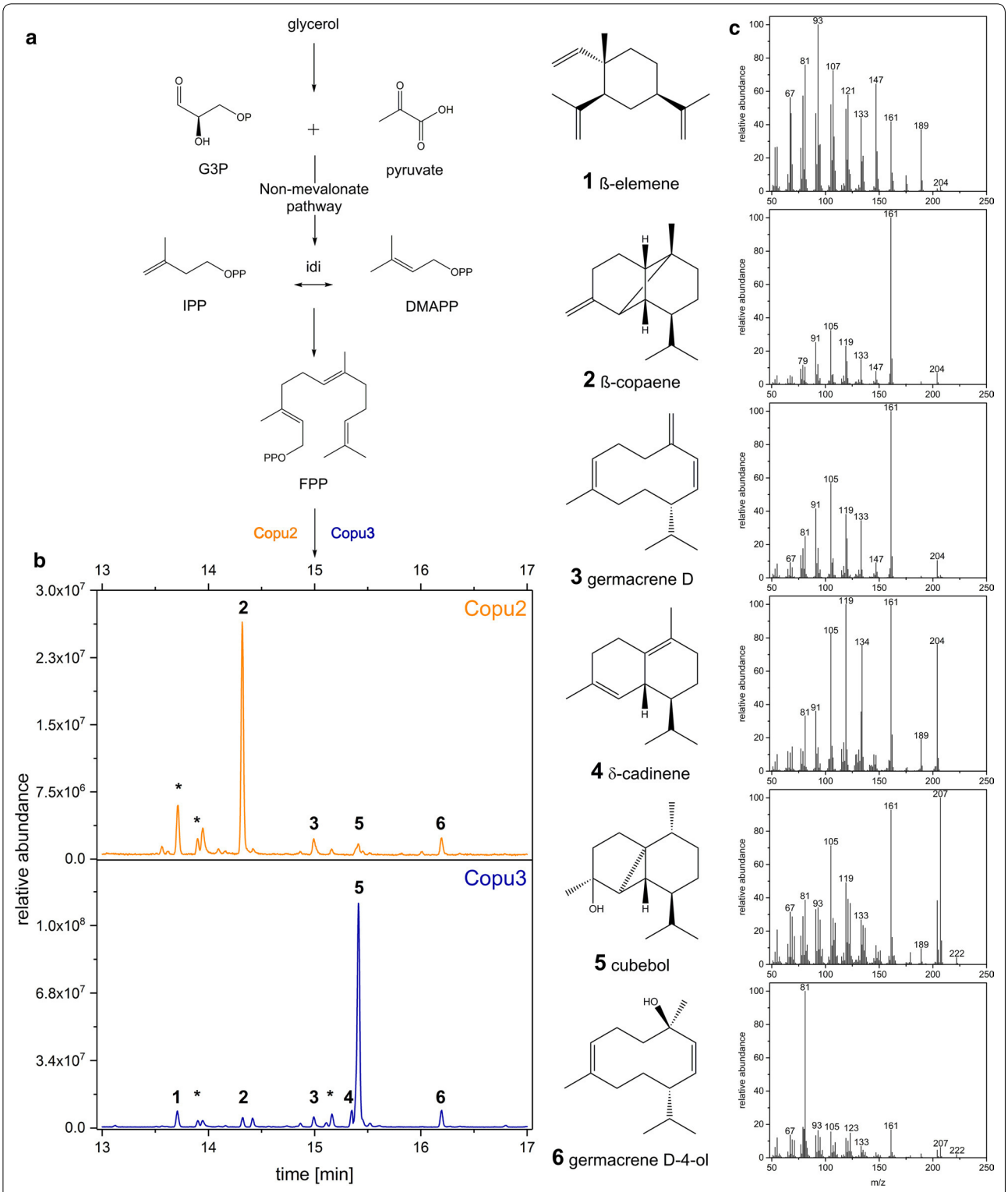

Fig. 2 The biosynthetic generation of β-copaene and cubebol. STP production relies on the precursor supply of the non-mevalonate (MEP) pathway (**a**). The respective GC–MS chromatograms of *E. coli* cultures co-expressing either Copu2 or Copu3 and the corresponding MEP bottleneck enzymes reveal new product peaks and distinct product profiles due to the specific cyclization reaction (**b**). The respective detailed MS spectra allow for a putative compound assignment (**c**). The asterisks refers to terpenes without any MS spectra match in the NIST database

A comparison of NMR spectra to reported references confirmed the presence of β-copaene [26, 27], cubebol [28] and δ-cadinene [29] as products of Copu2 and Copu3, respectively. Based on the NMR data, we designated Copu2 as a new β-copaene synthase. Conversely, Copu3 was designated as a new, highly selective cubebol synthase.

Interestingly, a rearrangement of β-copaene to the better described α-isomer could be observed in this context. The complete conversion took place by storing the pure compound in chloroform for < 12 h (Fig. 3). α-Copaene has been shown to possess important biological properties, including anticarcinogenic as well as antioxidant activity in the field of neurodegenerative diseases [30, 31], or serves as an insect attractant [32].

Phylogenetic synthase evolution

At present, the identification and characterization of terpene cyclases from the group of Basidiomycota is limited. Protein sequence-based phylogenetic analysis of the 29 genetically and biochemically characterized STPSs derived from Basidiomycota revealed four distinct clades (clade I–IV) (Fig. 4). The clustering by sequence conservation suggests that STPSs within one specific clade may catalyze the same or a related cyclization reaction. It also revealed that Copu2 and Copu3 clustered in clade I together with all other Basidiomycota-derived STPSs, generating either β-copaene or cubebol (ACTPS9, Cop4 and Stehi_128017). Additionally, most enzymes that generated cadinene isoforms clustered in clade I. For several candidate enzymes constituting this clade (Cop4, Omp4, Omp5a and 5b), a substrate cyclization mechanism has already been postulated, involving a 1,10-cyclization of (3R) nerolidyl diphosphate (NPP). The conversion of initial FPP involves the formation of a cis-germacradienyl cation, followed by a subsequent 1,6-cyclization. The final result is various STPs derived from a cadinyl cation (Fig. 5) [14, 33, 34]. By contrast, the clade II STPS mechanism involves a 1,10-cyclization of (2E,6E)-FPP to an E,E-germacradienyl cation (Omp1–3, Cop 1–3) [35], generating predominantly α-muurolene and germacrene A as well as different types of cadinol. Clade III STPSs share a 1,6-cyclization mechanism of (3R)-NPP or (3S)-NPP, leading to a bisabolyl cation [33, 36], forming mainly barbatene or α-cuprenene. Finally, clade IV STPSs share a common 1,11-cyclization mechanism of (2E,6E)-FPP [37]. Except for GME9210, all enzymes in this clade exclusively represent functionally characterized Δ6-protoilludene synthases.

In silico homology modeling [38] of Copu1-3 as well as Cop4 and structural alignments with the selinadiene cyclase (4OKM), a monomeric STPS from *Streptomyces pristinaespiralis*, as a nearest neighbor reference structure were carried out (see Additional file 1). Interestingly, the sequence identity analyses strictly differentiates sequences from distinct organisms while the structural analysis (RMSD calculation) reveals a close structural relation between Copu3 and Cop4. Both enzymes produce δ-cadinene, β-copaene and cubebol.

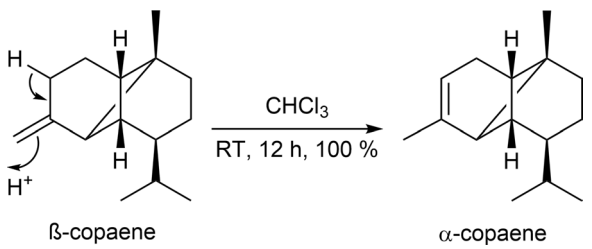

Fig. 3 Reaction scheme to illustrate the rearrangement from β-copaene to α-copaene. This reaction is observed in the presence of chlorophorm

Technical scale, fed-batch production of β-copaene and cubebol

To investigate the production performance of Copu2 and Copu3 in an optimized microbial system, fed-batch fermentation experiments were carried out in 1.3-L fermenters. This production scale provided for technically relevant amounts of β-copaene and cubebol. *E. coli* HMS174 (DE3) strains co-expressing either Copu2 or Copu3 and the corresponding MEP bottleneck enzymes were cultured at 30 °C under controlled conditions (Fig. 6). The Copu2-expressing culture reached its stationary phase after 46 h with a final calculated OD_{600} of 130, providing a final β-copaene titer of 215 mg/L. Based on these data, the specific β-copaene production and productivity were 4.4 mg/g dry cell weight (DCW) and 4.7 mg/L/h, respectively. To the authors' knowledge, this is the first report of any quantitative biotechnological production of β-copaene. In comparison, the Copu3-based fermentation generated a cubebol titer of 497 mg/L (calculated OD_{600} of 182), and the specific cubebol production and productivity even reached 7.2 mg/g DCW and 11.2 mg/L/h, respectively. The reported titers in this study exceeded concentrations of alternative approaches obtained by equivalent fermentations based on a plant-derived cubebol synthase (titers of 10 mg/L cubebol) [48] by 50-fold.

Discussion

In this study, three new terpene synthases (Copu1-3) were identified from genome data of the Basidiomycota *Coniophora puteana* and functionally characterized for their product profiles. At present, this filamentous fungus has only been described in the context of cellulytic activity. Therefore, this is the first report of any enzyme

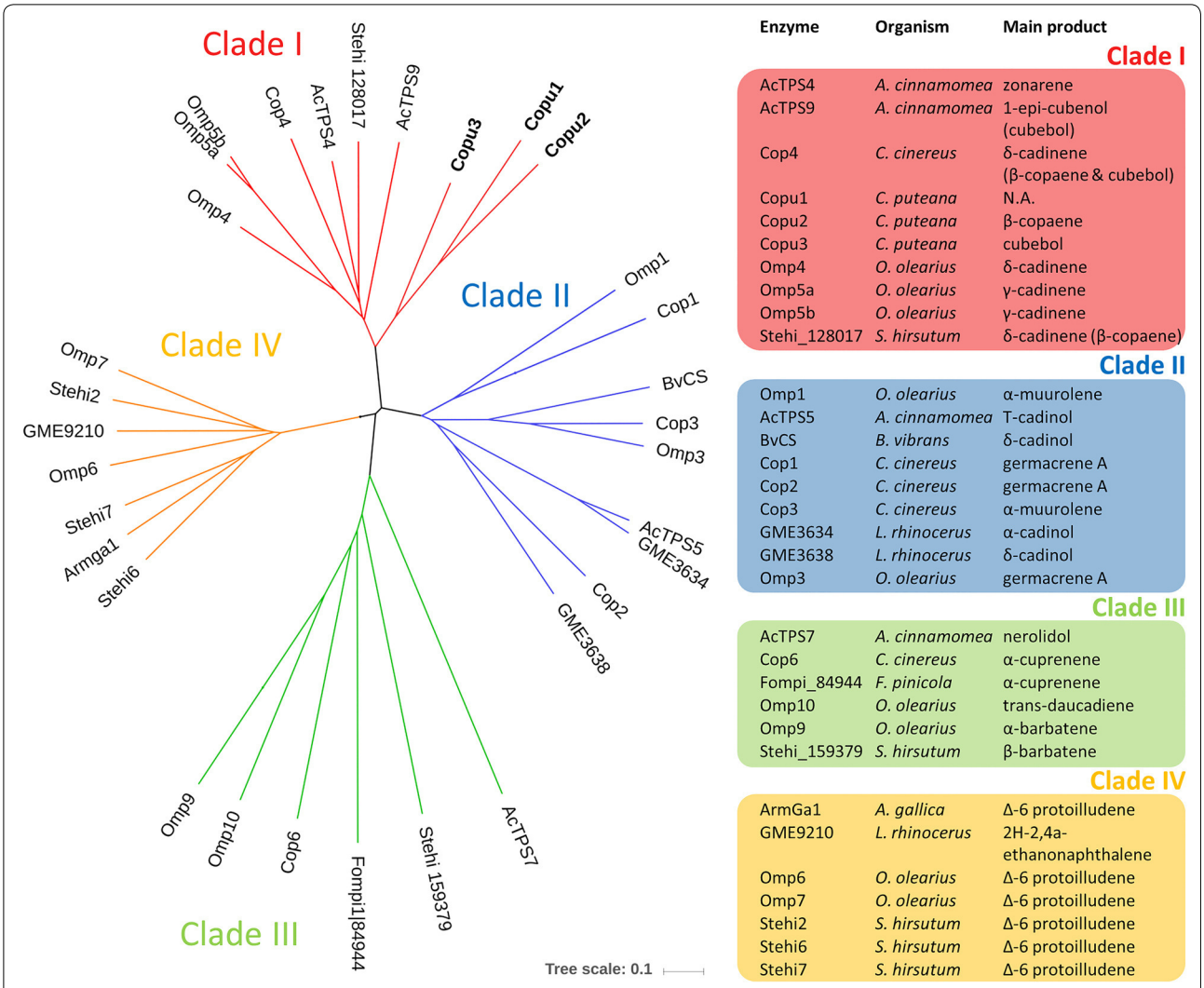

Fig. 4 Unrooted neighbor-joining phylogram of all 29 reported and experimentally characterized STPSs from Basidiomycota. The protein sequence based analysis includes STPSs from *Antrodia cinnamomea* [39], *Armillaria gallica* [40], *Boreostereum vibrans* [41], *Coprinopsis cinereus* [35], *Fomitopsis pinicola* [42], *Lignosus rhinocerus* [43] *Stereum hirsutum* [37], *Omphalotus olearius* [42] as well as *Coniophora puteana*. The phylogenetic analysis revealed four distinct clades, divided into different cyclization mechanisms and product profiles. Copu1-3 belong to clade I including all other known β-copaene or cubebol synthases from Basidiomycota. For detailed information about the used sequences and accession numbers, see Additional file 1

system involved in secondary metabolite biosynthesis from this organism. Only a few reports describe the successful functional characterization of TPSs from the phylum of Basidiomycota [35, 37, 39, 42]. In this study, the heterologous expression of Copu2 and Copu3 functionally confirmed their identities as sesquiterpene synthases (STPSs). By contrast, Copu1 expression in either an engineered sesqui- or diterpene *E. coli* production system did not yield any products. The Copu1 sequence may represent a non-functional TPS variant. This could be attributed to the fact that Basidiomycota tends to have very intron-rich genomes, and various functional transcripts may be generated by alternative splicing events. The in silico identification of the annotated Copu1 coding sequence may only encompass splicing events that result in a catalytically inactive enzyme [14, 49].

The selectivity of terpene synthases varies significantly in this enzyme class. In that respect, highly selective TPSs only provide a single product (e.g., the (+)-d-cadinene synthase from *Gossypium arboreum* [46]), while more promiscuous enzymes can generate in excess of 50 different compounds (e.g., the γ-humulene synthase (*Abies grandis*) [45]). Product promiscuity is prevalent, especially within the group of STPSs [50]. However,

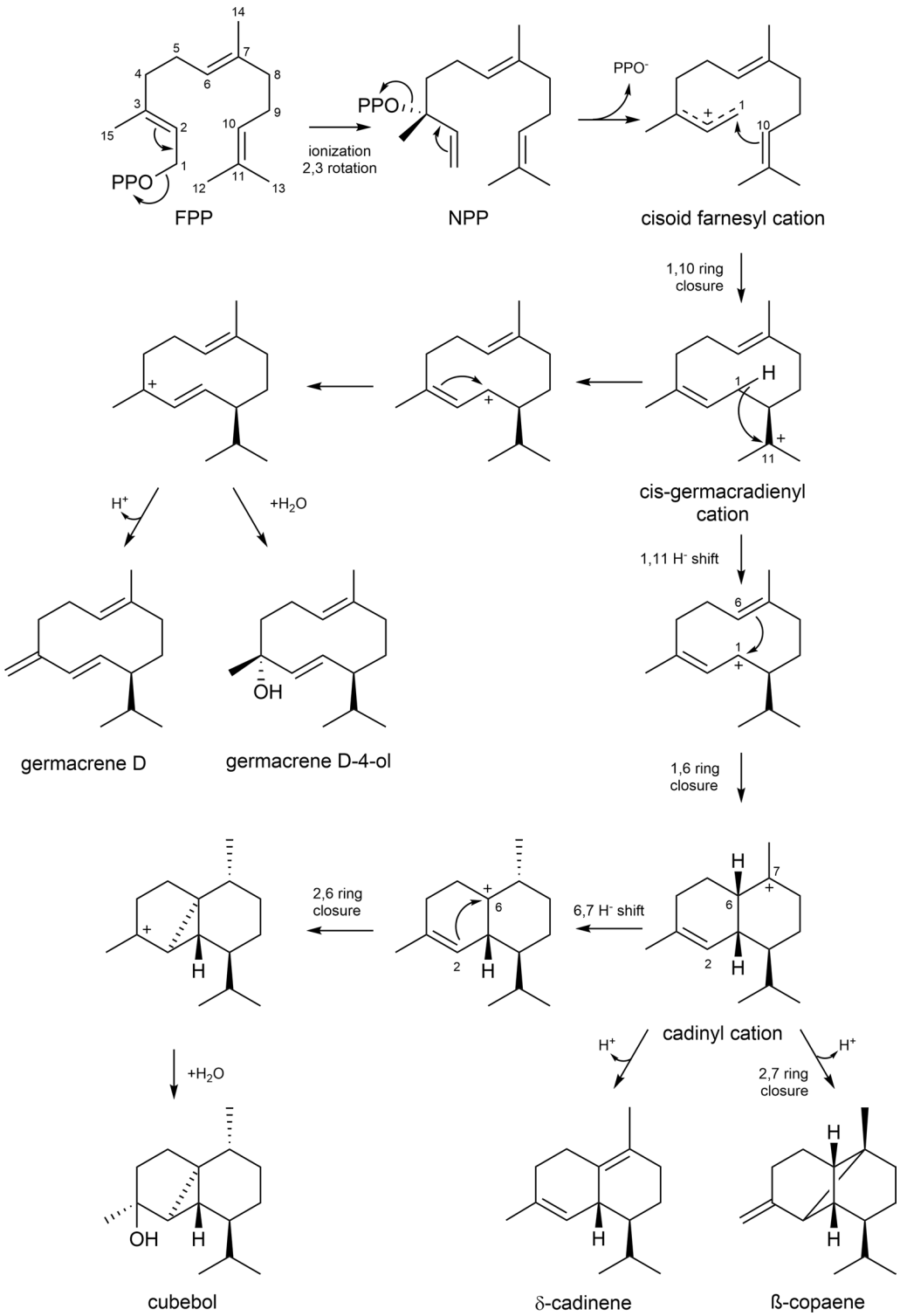

Fig. 5 Proposed cyclization mechanisms for the generation of the main STPs from Copu2 and Copu3. The reaction starts by ionization and isomerization of farnesyl diphosphate (FPP) creating nerolidyl diphosphate (NPP). The first cyclization is achieved by the formation of the C1–C10 bond. Subsequent C6–C1 bond formation yields a cadinyl cation, which represents a general precursor for various cadalane and cubebane-type STPs [14, 44–47]

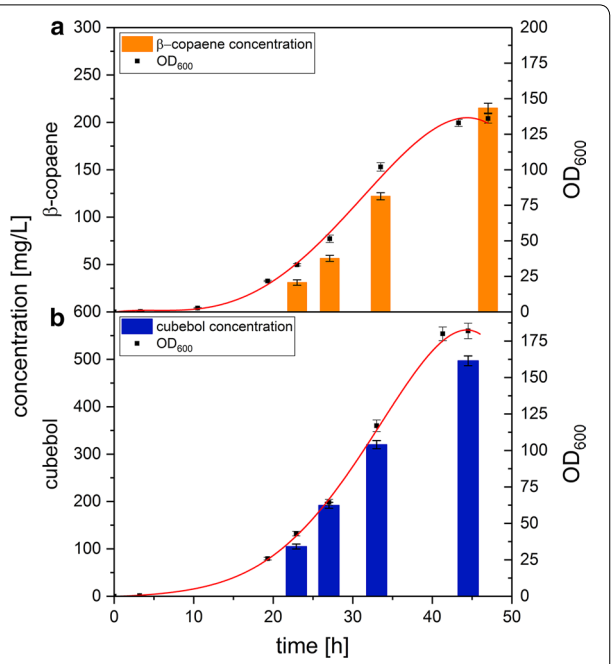

Fig. 6 *E. coli* growth curves and time-dependent concentration of β-copaene and cubebol in controlled fed-batch fermentations. Expression of Copu2 (**a**) and Copu3 (**b**) in *E. coli* resulted in a constant concentration increase, which was finally limited by the stationary phase. The error bars represent the mean values ± standard deviation of technical triplicates

heterologously expressed Copu2 and Copu3 display a fairly restricted product profile. As β-copaene accounted for 62% of all terpenoid products in Copu2 fermentations, this enzyme was identified as a β-copaene synthase. By contrast, cubebol was the main product (75%) of Copu3 fermentations, which designated this enzyme as a cubebol synthase. To the best of our knowledge, this is the first report of a selective β-copaene synthase and cubebol synthase. Both enzymes combine high product titers with significant product selectivity. Although we cannot completely rule out an *E. coli*-specific effect on the generated products, we believe that the enzymatic reaction corresponds to the natural conditions in *C. puteana*.

To provide insights into the structural and functional diversity of STPSs from Basidiomycota, we carried out a comprehensive phylogenetic analysis. This analysis encompassed the STPS sequences of *Antrodia cinnamomea* [39], *Armillaria gallica* [40], *Boreostereum vibrans* [41], *Coprinopsis cinereus* [35], *Fomitopsis pinicola* [42], *Lignosus rhinocerus* [43] *Stereum hirsutum* [37] and *Omphalotus olearius* [42], which are all functionally characterized. The phylogenetic analysis suggests that Copu1-3 belong to the same STPS clade, sharing a 1,10-cyclization mechanism with other related enzymes in this group (Fig. 4). Clade I encompasses all previously reported Basidiomycota STPSs that are capable of generating β-copaene, cubebol or cadinenes, which are all derived from a cadinyl cation intermediate (Fig. 5). However, Copu2 and Copu3 are the only STPSs realizing the generation of significant amounts of β-copaene and cubebol, respectively. Both enzymes form germacrene D and germacrene D-4-ol as minor products, which are derived from an earlier cis-germacradienyl cation intermediate. This carbocation intermediate is present in the biosynthesis of various cadinene type compounds [51]. The phylogenetic analysis based on amino acid sequences clearly illustrates the potential for possibly predicting enzymatic reaction mechanisms and an eventual product profile. Phylogenetic tree generation based on structural information might further improve this functional in silico prediction of an unknown terpene synthase. However, for a more precise prediction of enzymatic functions, more data have to be generated for Basidiomycota since this research area is still at a very early stage and comprises only a few candidate enzymes.

Cubebol is of interest to the cosmetic and flavor industry as it is already a registered product with a pronounced cooling effect [21] and is therefore formulated into dietary supplements and flavoring agents [9, 20]. Also the performed conversion of β-copaene to the more extensively investigated α-isoform opens up access to a potential pharmaceutical market, based on the proposed anticarcinogenic as well as antioxidant activity in the field of neurodegenerative diseases [30, 31]. At present, both compounds are commercially extracted from plant material. Specifically, β-copaene is a component of the essential oil (15%) from *Piper nemorense* leaves [52]. Similarly, cubebol is a constituent in the berry oil (5–10%) of *Piper cubeba* [53, 54] or the volatile leaf oil (3.3%) extracted from *Juniperus convallium* [55]. The quality and quantity of the target compounds from plant extracts vary seasonally and with global production location [53]. Consequently, purification from these natural extracts is technically challenging and cost-intensive [11, 12] because of various isoforms and structurally related impurities in complex mixtures. In contrast to the plant extracts, the biotechnological production of both β-copaene and cubebol would provide an efficient and sustainable alternative with simplified purification procedures. This could ensure a constant product quality, which is of high interest to the flavor industry [12].

The biotechnological formation of cubebol or β-copaene could only be provided by very few characterized STPSs. The δ-cadinene synthase VvPNCuCad (HM807407.1) from grapevine (*Vitis vinifera*) encoding a multi-product STPS shows 20.5% cubebol selectivity when expressed in *E. coli* [56]. The fungal δ-cadinene

synthase Cop4 (A8NU13.1) from *C. cinereus* is also designated as a cubebol and β-copaene synthase. The product profile of recombinant Cop4 is reported to generate 30% β-copaene and 10% cubebol with respect to the total terpene production titer [35]. Even under optimized in vitro conditions, Cop4 does not generate in excess of 34.2% cubebol [47]. Other fungal STPSs with related enzymatic activity have been cloned and characterized from *A. cinnamomea* (AcTPS9) [39] and *S. hirsutum* (Stehi_128017) [37] but with significantly lower production rates. Even though several other plant enzymes are listed for the more common α-copaene isoform [57, 58], Cop4 represented the only known enzyme with a relevant formation of β-copaene to date. Therefore, Copu2 and 3 isolated from *C. puteana* are the only enzymes capable of the highly selective generation of their main products β-copaene (62% product selectivity) and cubebol (75% product selectivity) and represent an excellent basis for biotechnological production.

The only described biotechnological production approach reported for the quantitative generation of cubebol utilized a patented plant enzyme (CQ813505.1 from grapefruit *Citrus x paradisi*; 28% cubebol selectivity) but only provided titers of 10 mg/L [48], which is well below the titers we report with Copu3 fermentations. In our *E. coli*-based approach, we were able to demonstrate cubebol titers of 497 mg/L even without significant optimization of the fermentation procedures. The production of 215 mg/L β-copaene represents the first biotechnological process with technically relevant titers of this compound. Our data expand the set of functionally characterized STPSs, which can be used for biotechnological processes. With respect to literature data and current state-of-the art technology of essential oil extraction [59, 60], our production system provided, within < 2 days, cubebol concentrations (w/w) that were similar to the natural producer (ripe berries of *piper cubeba*) [53] over a whole season. At the same time, it represents a 50-fold increase compared to the only reported target-oriented biotechnological approach in yeast [48].

Conclusion

Fungi have an enormous capacity for biosynthesis of versatile natural terpenoids and therefore represent an outstanding resource of new metabolic pathways for biotechnological production. Regarding the increasing availability of complete fungal genomes, the quantity of genetic information is expanding continuously. However, the complex biochemical characterization of the in silico-annotated enzyme activities is lagging far behind. While most enzyme-focused studies involve Ascomycota-derived sequences, the biosynthetic diversity of secondary metabolites, particularly related to the terpenome of Basidiomycota, is largely unexplored.

In this study, we focused on the discovery and investigation of sesquiterpene synthases (STPSs) from the Basidiomycota *Coniophora puteana*. We identified the STPSs Copu1-3, which were subsequently expressed in an engineered *E. coli* host capable of either sesqui- or diterpene production. While the expression of Copu1 did not show any terpene accumulation, the sesquiterpene β-copaene was the main product of Copu2. Hence, Copu2 was designated as the first exclusive β-copaene synthase (62% product selectivity). Similarly, Copu3 was identified as the most efficient cubebol synthase (75% product selectivity) to date. The metabolic optimization of a microbial production host, including the introduction of MEP pathway enzymes and the fungal enzymes Copu2 and Copu3, created microbial cell factories for the de novo production of 215 mg/L β-copaene and 497 mg/L cubebol. Although further work is needed to optimize the product titers, the current whole-cell systems could serve as a promising basis for the development of large-scale biotechnological production of these compounds. Patent filed.

Methods

Gene cloning, plasmid construction and culture condition

Escherichia coli strain DH5α was used for cloning and *E. coli* strain HMS174 (DE3) for terpene production. Genes encoding for the sesquiterpene synthases Copu1 (327 AA; XP_007772164.1); Copu2 (340 AA; XP_007771895.1) and Copu3 (332 AA; XP_007765978.1) from *Coniophora puteana* were ordered codon-optimized (Eurofins Genomics) for improved efficiency in *E. coli* and cloned into a pACYC-based expression vector system. The final production construct contained a single operon with selected bottleneck enzymes of the MEP pathway (DXS; 1-deoxy-D-xylulose-5-phosphate synthase from *E. coli*; WP_099145004.1) (idi; isopentenyl pyrophosphate: dimethylallyl pyrophosphate isomerase from *Haematococcus lacustris*; AAC32208.1) and the corresponding cyclase under the control of a lac-I-derived constitutive promoter [61]. Cultures were grown in modified R-media [62] (13.3 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 5.0 g/L yeast extract, 35 g/L glycerol, 4.9 mL/L 1 M $MgSO_4$, 2.45 mL/L 0.1 M Fe(III) citrate, 10 mL/L 100 × trace elements solution (5 g/L EDTA, 0.83 g/L $FeCl_3$–$6H_2O$, 84 mg/L $ZnCl_2$, 13 mg/L $CuCl_2$–$2H_2O$, 10 mg/L $CoCl_2$–$2H_2O$, 10 mg/L H_3BO_3, 1.6 mg/L $MnCl_2$–$4H_2O$), 1 mg/L Thiamin) supplemented with the appropriate antibiotics, ampicillin (100 µg/mL) or chloramphenicol (34 µg/mL), at 30 °C and 90 rpm shaking.

Terpene isolation

For analytical terpene isolation within the screening process, 35 mL of the selected cultures was mixed with 15 mL of an extraction solution (ethyl acetate, hexane and ethanol; 1:1:1). The suspension was then strongly shaken for 15 min followed by a 60 s centrifugation step at 8000*g* for phase separation. A defined volume of the upper organic phase was then sampled and analyzed by GC–MS.

Fermentation and preparative extraction

All fermentations were performed in a DASGIP® 1.3 L parallel reactor system (Eppendorf AG) using modified R-media [62] supplemented with 35 g/L glycerol. An overnight preculture was used for inoculation (OD = 0.1). The cultivation temperature was kept constant at 30 °C. The initial stirrer velocity and airflow were set to 200 rpm and 0.5 volumes of air per volume of medium per minute (vvm), respectively. The dissolved oxygen (DO) was kept at 30% and controlled by stirrer velocity (up to 1000 rpm), oxygen content (up to 100%) and airflow. A pH value of 7.00 was maintained by adding 25% sodium hydroxide solution as needed. A pH-based feed was activated by pH values exceeding 7.05, which triggered a feed shot of 40 mL. The feed solution consisted of 600 g/L glycerol, 20 g/L $MgSO_4 \cdot 7H_2O$, 15 mL/L 100 × trace elements solution (5 g/L EDTA, 0.83 g/L $FeCl_3$–$6H_2O$, 84 mg/L $ZnCl_2$, 13 mg/L $CuCl_2$–$2H_2O$, 10 mg/L $CoCl_2$–$2H_2O$, 10 mg/L H_3BO_3, 1.6 mg/L $MnCl_2$–$4H_2O$), 70 g/L collagen hydrolysate and 7.5 g/L yeast extract (pH = 7.00). Samples were taken regularly to measure the OD_{600} and the respective terpene concentration.

The whole cultivation broth was extracted by adding an equal volume of ethyl acetate and ethanol (1:1) to the cell culture. The suspension was shaken for 12 h at 22 °C followed by a centrifugation step for 15 min at 7000*g*. After separating the supernatant from the pellet, an additional 1/4 volume of hexane was added. The mixture was shaken for 2 h before the upper phase was isolated using a separation funnel. The organic layer was concentrated using a rotary evaporator. The remaining crude oil containing terpenoids was dissolved in an ACN and H_2O (9:1) solution for further HPLC purification.

Terpene purification, identification and quantification

The purification was performed by preparative HPLC using a NUCLEODUR® C18 HTec 250/10 mm 5 µm column (MACHEREY–NAGEL GmbH & Co. KG) and a diode array UV detector at 2.2 mL/min flow rate. The injection volume was 2 mL at a concentration of 25 mg/mL of crude extract in ACN and H_2O (9:1). The separation was performed applying an ACN gradient starting at 90% and increasing to 100% within 10 min. This was maintained for 30 min. The oven temperature was set to 30 °C. The terpene peaks were detected at 210 nm wavelength. Fractions containing the pure product, determined by GC–MS analysis, were pooled and concentrated using a rotary evaporator.

Escherichia coli whole-cell conversion extracts from *C. puteana* Copu1-3 were analyzed by a Trace GC Ultra with DSQ II (Thermo Scientific). The sample was loaded by TriPlus AS onto an SGE BPX5 column (30 m, I.D. 0.25 mm, film 0.25 µm). The initial oven temperature was set at 50 °C for 2 min, increased to 320 °C at a rate of 10 °C/min, and held for 3 min. MS data were recorded at 70 eV (EI) and m/z (rel. intensity in %) as total ion current (TIC). Data were collected in full scan mode (m/z 50–650). Structural determination of terpenes was conducted by comparison to mass spectra data of the NIST Standard Reference Database. Concentrations were quantified by correlating the FID peak area to a defined α-humulene and cubebol standard of known quantity (see Additional file 1 for calibration curves).

The NMR spectra of the products were recorded in $CDCl_3$ (cubebol and δ-cadinene) or C_6D_6 (β-copaene) with a Bruker Ascend™ 400 MHz NMR spectrometer. All chemical shifts are relative to $CDCl_3$ at δ = 7.26 (1H-NMR) and $CDCl_3$ at δ = 77.16 (13C-NMR) or C_6D_6 at δ = 7.16 (1H-NMR) and C_6D_6 at δ = 128.06.16 (13C-NMR) using the standard δ notation in parts per million.

Protein modeling

The webservice RaptorX was applied for homology modeling studies (http://raptorx.uchicago.edu) [63]. Subsequently, the predicted structures (Cop4, Copu 1-3) were analyzed by Visual Molecular Dynamics (VMD) (http://www.ks.uiuc.edu/Research/vmd/) [64]. Protein structures were aligned and compared using the MultiSeq [65], and scenes rendered by Tachyon [66], both, implemented in the VMD software package. Phylogenetic trees of these five proteins were calculated using sequence identity or the rmsd value as tree generation criteria.

Structure function analyses of Copu3 were performed as previously described [38].

Phylogenetic analysis

A multiple sequence alignment was generated with Clustal Omega (https://www.ebi.ac.uk/Tools), using seeded guide trees and HMM profile techniques. Evolutionary analyses were conducted with iTOL (https://itol.embl.de/). The phylogenetic tree was inferred using the maximum likelihood method.

Additional file

Additional file 1. Details on phylogenetic analysis, sequence homology, protein structure modelling and additional NMR data are provided as additional information.

Abbreviations

AA: amino acid; DCW: dry cell weight; DMAPP: dimethylallyl pyrophosphate; FPP: farnesyl pyrophosphate; GC–MS: gas chromatography–mass spectrometry; IPP: isopentenyl pyrophosphate; MEP: non-mevalonate pathway; NIST: National Institute of Standards and Technology; NPP: nerolidyl diphosphate; STP: sesquiterpenoid; STPS: sesquiterpene synthase; TPS: terpene synthase.

Authors' contributions

Conceived the project: WM and MH. Designed and performed the experiments: WM. Analyzed the data: WM and MH. Prepared the manuscript: WM and TB. Supervised the whole work: TB, MF, and NM. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file. Additional data required is available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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6 CYP-SWINGER: NEW STRATEGIES FOR THE EFFICIENT OXO-FUNCTIONALIZATION OF DITERPENES BY NEW-TO-NATURE OXYGENASE-REDUCTASE COMBINATIONS

Initial Research by

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6.1. SUMMARY

Chemical functionalization of hydrocarbon backbones usually mediates essential bioactive functions and extends the spectrum of action for a variety of natural substances. Cytochrome P450 monooxygenases (CYPs) provide the most common modifications by activation and incorporation of additional oxygen atoms to their substrates.[161] However, despite all progress in synthetic biology, the realization of this enzymatic functionalization remains a challenge for whole cell biocatalysis. Due to the membrane-bound characteristics of P450 systems, bacterial production platforms as *E. coli*, are limited as heterologous host.[162] Additionally, the required presence of a co-localized adequate Cytochrome P450 reductase (CPR) partner, which provides essential electron equivalents for the reaction cascade, represents a further challenge.[163, 164] The recreation of biosynthetic pathways for complex oxidized terpenoids in heterologous hosts faces numerous challenges like optimizing the expression of CYPs and CPRs as well as balancing the redox environment within the cell.[152, 165] Therefore, the functional expression of P450 systems remains a key element in the development of microbial platforms for biosynthesis of complex isoprenoids. The expression of translational CYP-CPR fusion proteins has proven to be a successful strategy in *E. coli* for an optimized electron transfer.[67, 166] However, these systems require considerable development effort and are typically not transferable to other substrates.

Within this project, the selective hydroxylation of the diterpene taxadiene was reconstructed and confirmed by utilizing a CYP-CPR fusion enzyme. First experiments revealed the successful replacement of the initial CPR unit from *Taxus baccata* by a different CPR from *Catharanthus roseus*, leading to an increased hydroxylation efficiency in *E. coli*. However, the general turnover rate of this system was still at a low level. Based on these results, a new strategy for efficient hydroxylation of different terpene structures in *E. coli* was evaluated. The basic principle was the separate expression of the individual redox partners without using the previously established protein linkers. This approach has a decisive advantage in expression efficiency due to the reduced size of the two individual enzymes compared to large fusion complexes. An optimized production plasmid allowed for the fast and efficient exchange of genetic sequences for different CYPs and CPRs. This approach resulted in the successful hydroxylation of taxadiene as well. Moreover, the biochemical pathway for the production of cylooctat-9-en-7-ol, including the corresponding flavin-dependent reductase-system Afr-Afx, was successfully implemented and tested within this setup. Subsequently, the exchange of CYP and CPR partners was carried out to investigate a

possible electron transfer within different non-native systems. The hydroxylation of taxadiene with the Afr-Afx system as well as the hydroxylation of cylooctat-9-en-7-ol via the *R. catharanthus* derived CPR was successfully verified. Therefore, it was concluded that electron transfer is not limited to native CYP-CPR systems, and P450 mediated reactions are possible although the electron transfer system was modified. This kind of enzymatic flexibility has not been discussed before. A screening of the different CYP-CPR systems with regard to their activity on non-native substrates revealed measurable activities for different combinations. However, the product yield was still too low for comprehensive structural analysis.[167]

Based on these results, the process was optimized for higher titers in order to enable further product characterization. First experiments focused on the direct biotransformation from purified terpenoids via engineered *E. coli* strains. A central aspect was the screening of different reaction parameters, such as number of cell cycles, temperature or solvents for extraction.[168]

The investigated system is still in an early development phase and needs further modification and optimization. Especially in the field of efficient and balanced protein expression, there is still a need for extensive research and adjustments. Nevertheless, the potential for a flexible but also efficient application is already evident at this stage.

7 Outlook

7.1. FUTURE TERPENE PRODUCTION

Facing a growing world population, limited supply of fossil resources as well as the progressing climate change,[1–3] the need and interest in microbial production of sustainable natural compounds and biofuels has risen.[3, 169] A broad replacement of petroleum derived synthetic chemicals with microbial fermentation products could lead to environmental benefits like reduced greenhouse gas emissions or waste streams. Simultaneously, the growing concerns about chemically produced ingredients and the public demand for 'bio-based' products will further promote microbial-derived natural compounds. Based on the attractive price margin of products from the pharmaceutical and fine chemicals sectors, many metabolic engineering companies focus on terpenoids, which are already applied and required in various areas.[130] The discovery of novel TPSs and modification enzymes offers the potential to initiate production of fragrances, drugs, biofuels and many other terpene-based commodities with currently unidentified bioactivities. However, the capacity of this biotechnological strategy depends on the identification of respective genetic tools, providing the desired compounds in sufficient amounts. Upcoming computational techniques and progress in system modelling as well as automated gene identification will extend this biotechnological toolbox and facilitate combinatorial assembly of biosynthetic pathways.

7.2. PROCESS OPTIMIZATION TOWARDS INDUSTRIAL IMPLEMENTATION

To reach future commercialization status, all biotechnological processes have to consider economic parameters in terms of costs, resources and scalability. Substantial investments and significant efforts are required to achieve economically attractive performance indicators in terms of titer, yield and productivity.[100, 130, 149, 151, 152, 170] Therefore, the development of microbial strains and fermentation strategies needs to progress constantly. Optimization of microbial production routes can be addressed by adjusting different parameters in almost every process step. Additional feeding of precursor molecules or an organic overlay for continuous product depletion may significantly increase the product output.[148, 153] External adsorption systems for simplified product purification and reduced cellular stress represent alternative extraction methods with great potential.[66] The choice of plasmid based systems or a genomic integration is of high importance for efficient strain development. Commonly used high-copy plasmids might suffer from instability, expression variability, and are limited in the number of expressed genes.[171] Recent developments in metabolic

engineering involve the combination of different promoters, RBS and gene sequences. Concurrently, simplified screening approaches allow for rapid identification and selection of the most suitable and productive genetic combinations.[67, 172]

Optimization of the isoprenoid pathway itself is perhaps one of the most direct ways to improve basic productivity. MVA- as well as MEP pathway offer different enzymatic steps for the possible manipulation in microbial hosts. Unbalanced expression of the involved pathway genes is a serious obstacle for sufficient product yields. Therefore, elimination of bottlenecks, pathway balancing and general operon design represent essential development strategies.[67, 76, 172, 173] By engineering the yeast-derived MVA isoprenoid pathway into *E. coli*, native regulatory elements are bypassed and the synergy of both essential pathways for the supply of DMAPP and IPP has been proven to be highly advantageous.[137, 174] At the same time, there are efforts to include artificial biosynthetic pathways, which result in robust yields. This leads to further possibilities with regard to future non-natural terpene scaffold and new chemical functionalities.[175]

7.3. STRUCTURAL MODIFICATION AND MICROBIAL CONSORTIUM

The heterologous production of complex oxidized terpenoids significantly depends on the evaluation of functional P450 systems. Although *E. coli* has been developed as a flexible and accessible production host, the functional expression of the required CYP and CPR enzymes represents an immense challenge.[152, 165] Most of the considered P450 systems depend on plant-based enzymes, which are membrane-bound and require the presence of an additional redox partner.[164] Therefore, *S. cerevisiae* is often preferred for the biotechnological production of hydroxylated compounds, due to the availability of basic native P450 systems and the presence of an endoplasmic reticulum.[176, 177]

Besides the challenging expression of plant enzymes in *E. coli*, the utilization of a few accessible and soluble P450s has been successfully demonstrated. BM-3 from *Bacillus megaterium* is one of the best-characterized bacterial P450 system, including an available crystal structure.[178] The CYP- and CPR- domains are provided by a single enzyme. Previous studies have demonstrated the impressive flexibility of this system, which is not limited to its natural catalytic function. Substrate- and product spectrum can be altered extensively by sequence mutagenesis.[179, 180]

A further promising approach for the specific production of hydroxylated isoprenoids represents the bioengineering of different individual microorganisms as a microbial network, instead of optimizing a single production host.[181] With advancing insight into

individual metabolic processes, the generation of coordinated microbial consortia might overcome complex biochemical tasks by separating complex pathways into shorter process units. Combination organism-specific advantages in terms of feedstock utilization, enzyme expression or provided reaction milieu facilitates various options for future terpenoid biosynthesis.[137, 182]

7.4. THE OUTSTANDING POTENTIAL WITHIN THE GENETIC CODE OF FUNGI

The realm of fungi is so far only poorly explored and hardly accessible for biotechnological approaches. Little is known about the responsible biochemical pathways, although they generate a huge diversity of natural products including various terpenoids.[99] Nevertheless, some terpene related enzymes have already been described, revealing TPSs as well as fungal P450 systems for further structural modifications.[99, 115] Fungal whole genome projects remain largely unexplored and involve numerous rarely characterized enzymes and putative TPSs.[99, 183] Since many currently accessible TPSs and P450 systems are still plant derived, which is a major challenge for microbial expression, the further intensified investigation of fungal resources would be of great advantage. Fungal systems are also promising for natural product discovery and engineering as the biochemical pathways are typically clustered within the genome.[184] These clusters share in common several TPSs, CYPs, and other backbone modifying enzymes.[185] Since required time and financial costs to elucidate and annotate whole genomes has drastically decreased in the last decade,[186] fungal genome mining combined with heterologous enzyme characterization has the potential to facilitate the discovery and biotechnological production of industrially relevant terpenes.[99]

Symptomatically, the genomic sequence data of the studied Basidiomycota *Coniophora puteana* contains more than 20 putative TPSs and only few have been investigated in experimental settings so far. However, the initial characterization and expression in *E. coli* revealed an efficient biosynthetic transfer and robust terpene production.[115] The excellent properties of the characterized enzymes indicate great opportunities in order to discover a variety of unknown enzymes for natural compound production.

7.5. EXPANSION OF THE ENZYMATIC PRODUCT PROFILE

Many studies have demonstrated the flexibility of TPSs by generating enzyme mutants with altered product profiles or completely new activities. Mutant libraries are created

via systematic variation of the enzymatic amino acid residues within the active site cavity to affect substrate specificity, product purity or catalytic efficiency. Most successful mutations lead to a broader product range and terpene diversity, providing insights into the mechanistic functions.[187] However, enzymes with a higher specificity towards a single main product are favored in an industrial environment. The targeted evolution of TPSs with a high degree of accuracy and tailor-made product profiles is therefore preferred.[188, 189] Further efforts in computer assisted protein engineering and high-throughput screening will lead to the identification of highly efficient TPSs mutants with superior product profiles.

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CHAPTER III
APPENDIX

9 ABBREVIATIONS

ACN	Acetonitrile
ATP	Adenosine triphosphate
CBT-ol	Cembratriene-ol
CPC	Centrifugal partition chromatography
CPR	Cytochrome P450 reductase
CYP	Cytochrome P450 monooxygenase
diTPS(s)	Diterpene synthase(s)
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DXS	1-deoxy-D-xylulose 5-phosphate synthase
EFSA	European Food Safety Authority
EtOAc	Ethyl acetate
EtOH	Ethanol
FPP	Farnesyl pyrophosphate
FPPS(s)	Farnesyl pyrophosphate synthase(s)
G3P	Glyceraldehyde-3-phosphate
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GGPP	Geranylgeranyl pyrophosphate
GGPPS(s)	Geranylgeranyl pyrophosphate synthase(s)
GPP	Geranyl pyrophosphate
GPPS	Geranyl pyrophosphate synthase
HPLC	High performance liquid chromatography purification
idi	Isopentenyl pyrophosphate isomerase
IPP	Isopentenyl pyrophosphate

IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
MEP	2-C-methyl-D-erythritol 4-phosphate
MVA	Mevalonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium acetate
NMR	Nuclear magnetic resonance
OD_{600}	Optical density at a wavelength of 600 nm
PCR	Polymerase chain reactions
RBS	Ribosome binding site
rpm	Revolutions per minute
SLM	Standard liter per minute
STPS(s)	Sesquiterpene synthase(s)
TPS(s)	Terpene synthases(s)
vvm	Volume of medium per minute

10 LIST OF FIGURES