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Biosynthesis, evolution and ecology of microbial terpenoids

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Terpenoids are the largest class of natural products recognised to date. While mostly known to humans as bioactive plant metabolites and part of essential oils, structurally diverse terpenoids are increasingly reported to be produced by microorganisms. For many of the compounds biological functions are yet unknown, but during the past years significant insights have been obtained for the role of terpenoids in microbial chemical ecology. Their functions include stress alleviation, maintenance of cell membrane integrity, photoprotection, attraction or repulsion of organisms, host growth promotion and defense. In this review we discuss the current knowledge of the biosynthesis and evolution of microbial terpenoids, and their ecological and biological roles in aquatic and terrestrial environments. Perspectives on their biotechnological applications, knowledge gaps and questions for future studies are discussed.

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1. Terpenoids – the most abundant secondary metabolites in nature

Terpenoids are a class of natural products that have attracted considerable research interest due to their vast abundance and large chemical diversity. Over 90 000 terpenoid compounds have been characterised (including steroids†),[1] making them the largest class of natural products. Terpenoids were first described as components of plant essential oils. Nobel laureate Otto Wallach first isolated and characterised the structures of several mono- and sesquiterpenes and described their reactivity and physical properties.[2] This pioneering work was performed at a time when NMR spectroscopy and X-ray crystallography were not available, and for structural elucidation scientists relied on chemical reactions and synthesis, which is a challenging task considering the structural complexity of terpenes with their often (poly)cyclic skeletons containing several stereogenic centers. It is well understandable that Wallach chose plants as sources of terpenoids; over the centuries plants have been used in traditional medicine to treat human diseases, attracting the interest of scientists to discover the active

† The Dictionary of Natural Products list steroids separately from terpenoids.

principles in plants. We now know that terpenoids occur in all kingdoms of life, including red algae,[3] land plants,[4] bacteria,[5] archaea,[6] fungi,[7] protists[8] and animals.[9,10] They are found in both terrestrial and aquatic organisms, and fulfil a wide range of both essential and specialised functions. These functions range from maintenance of the cell membrane integrity, stress alleviation, photoprotection to the attraction or repulsion of organisms and plant growth promotion and defense. This wide diversity of functions is mirrored by an even wider structural diversity of compounds. Microorganisms have evolved two different biosynthetic pathways, the methylerythritol 4-phosphate (MEP) and the mevalonate (MVA) pathways to form the basic building blocks of terpenes which are fused to oligomers that can be further converted into a range of different molecules by a single terpene synthase (TPS). Terpenoids are highly relevant to humans for their application as pharmaceuticals, fragrances, flavourings, colourants, pesticides and biofuels, among others.[11] In this review, we first describe general concepts of terpene biosynthesis and evolution, highlighting some of the most ubiquitous microbial terpenoids, such as geosmin and 2-methylisoborneol (2-MIB). We focus on the biological and ecological roles of microbial terpenoids in nature and provide an overview of workflows that are available to obtain functional insights into bioactive microbial terpenoids. We end with discussing the importance of and the knowledge gaps in the study of the industrially and medicinal relevant terpenoid compounds. For further reading, we refer to excellent recent review articles, which focus on bacterial[12] and fungal[13]

Mariana Avalos obtained her PhD studying the role of Streptomyces *volatile compounds, at the Institute of Biology, Leiden University. She then continued as a postdoc at the Netherlands Institute of Ecology (NIOO-KNAW), where she studied oxalotrophic bacteria and their role in the protection of plants against fungal infections. She has thus obtained a strong affinity for host–microbe and microbe–microbe interactions, and the major focus is currently on Actinobacteria and the role of volatile compounds, including terpenes, in the environment. She also has a strong affinity for research coordination, and she currently combines these two positions at Leiden University.*

Paolina Garbeva is a senior scientist at the Department of Microbial Ecology at The Netherlands Institute of Ecology. The focus of her group is to understand the fundamental mechanisms of microbe–microbe and plant–microbe communication with particular attention to the role of microbial volatiles. Her research group was among the first to reveal, the importance of volatiles in the long-distances microbial interactions (including fungi, bacteria and protists), as signaling compounds for communication or as suppressive agents in interference competition. Her research also provided new insights about the importance of volatile compounds in long-distance interactions between soil microorganisms and plants, and their possible agricultural application.

Lisa Vader is a graduate student at Utrecht University in Bioinformatics and Biocomplexity. In 2020 she obtained her BSc in Biology cum laude *at Leiden University, focusing on the role of terpenes in bacterial stress under the supervision of Prof. Gilles van Wezel. She is currently specializing in microbial genomics and is interested in studying the role of plasmids in antibiotic resistance and virulence of pathogens.*

Gilles van Wezel is Professor of Molecular Biotechnology and Scientific Director at the Institute of Biology, Leiden University. He is also distinguished professor at the Netherlands Ecology Institute (NIOO-KNAW) in Wageningen. He studied Chemistry at the Free University in Amsterdam and obtained his PhD in Biochemistry at Leiden University in 1994, on the control of protein biosynthesis in Streptomyces. *He did his postdoc at the John Innes Centre in Norwich with Prof. Mervyn Bibb, on catabolite repression. He became assistant professor in Leiden, working on* Streptomyces *cell division, and full professor in 2010. He co-founded the Biotech company Mycobics BV in 2001. His current research focuses on the biology of Actinobacteria and on approaches to discover novel antibiotics and anticancer compounds for clinical application.*

TPSs, bifunctional TPSs,[14] special aspects of diterpene biosynthesis,[15,16] the biosynthesis of non-canonical terpenoids,[17] the structural biology of TPSs,[18] and computational approaches for the understanding of terpenoid biosynthesis.[19,20]

2. Terpenoid biosynthesis and structural diversity

Terpenoid biosynthesis proceeds through three steps (Scheme 1A). During the first step, the terpene monomers are formed and then fused to yield oligomers through pathways that can be considered as primary metabolism. The second step is characterised by cyclisation reactions catalysed by TPSs to make up a large variety of terpene skeletons of low functionalisation.[12,18] Depending on the number of cyclisation events, only one or a few olefinic double bonds may be present in the final molecule, eventually in addition to an alcohol or sometimes ether function, if water is incorporated. Hydrocarbons arising from this step, which are formally oligomers of isoprenes, are terpenes *sensu strictu* and can be classified based on the number of monomer units they are derived from. For historical reasons, compounds arising from one unit are termed hemiterpenes, two units make up the monoterpenes, followed by sesquiterpenes (three units), diterpenes (four units), sesterterpenes (five units), triterpenes (six units) and tetraterpenes (eight units). During the third step, "tailoring enzymes" such as cytochrome P450 monooxygenases, dehydrogenases, reductases and/or transferases introduce oxidative and other modifications, sometimes associated with skeletal rearrangements or cleavage of groups.[21,22] These steps lead to the so-called terpenoids, a term that should be strictly separated from "terpenes". Terpenes are nonpolar and volatile (with decreasing volatility according to the number of carbon atoms),[23] while terpenoids are associated with increased polarity, *i.e.* water-solubility, and thus lower volatility. These functionalisation steps are often associated with increased bioactivity (*e.g.* as antimicrobials), as it allows for specific binding to biological target structures such as enzymes or the ribosome. In this review, we use "terpenoid" as a general term, while "terpene" is used only for compounds which fulfil the above-mentioned definition.

Despite the large number of known different compounds all terpenoids originate from only two building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Scheme 1B). They can be generated either from three units of acetyl-CoA *via* the classical mevalonate (MVA) pathway, mostly present in eukaryotes and, in a modified form, in archaea, or from pyruvate and glyceraldehyde-3-phosphate *via* the methylerythritol 4-phosphate (MEP) pathway, found in most bacteria and in the plastids of plants.[24,25] The MEP pathway, which consists of seven enzymatic steps, is the singular route to IPP and DMAPP biosynthesis in most bacteria.[26,27] The MVA pathway is also found in some species, including the Gram-positive cocci *Staphylococcus aureus* and *Streptococcus pneumoniae*, the spirochaete *Borrelia burgdorferi* and Gram-negative Myxobacteria. In a few bacteria both pathways are present, such as in *Listeria monocytogenes* and some *Streptomyces* strains.[28] In *Streptomyces* there is evidence that essential terpenoids are produced by the MEP pathway, while more specialised terpenoids such as antibiotics are produced by the MVA pathway.[28,29] A few obligate parasitic bacteria possess neither pathway, presumably because they can obtain their terpenoids from infected host cells.[30]

DMAPP and IPP show an interesting balanced reactivity, *i.e.*, the allyl diphosphate DMAPP is electrophilic at C1, while the homoallyl diphosphate IPP can attack DMAPP with its electron-rich C=C double bond as a nucleophile, leading to a tertiary cationic intermediate that is sufficiently stabilised by hyperconjugation. A subsequent stereospecific deprotonation with loss of the 2-*pro-R* proton completes their fusion to geranyl diphosphate (GPP, C10) as the precursor to all monoterpenes (Scheme 1B).[31] This reaction is catalysed by an oligoprenyl diphosphate synthase, an enzyme from the prenyltransferase family. Subsequent further elongation steps with IPP lead to farnesyl diphosphate (FPP, C15), the precursor of

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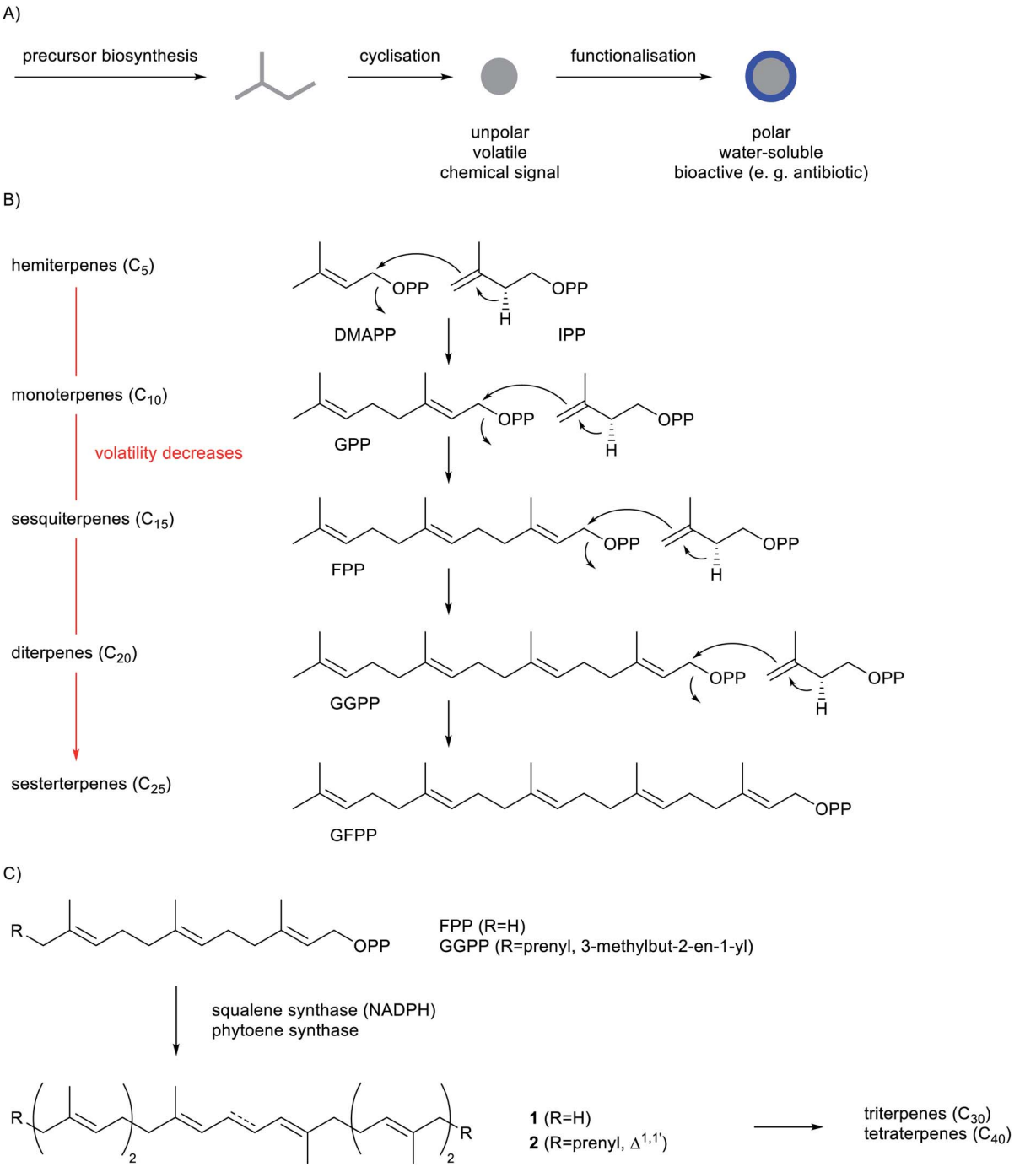
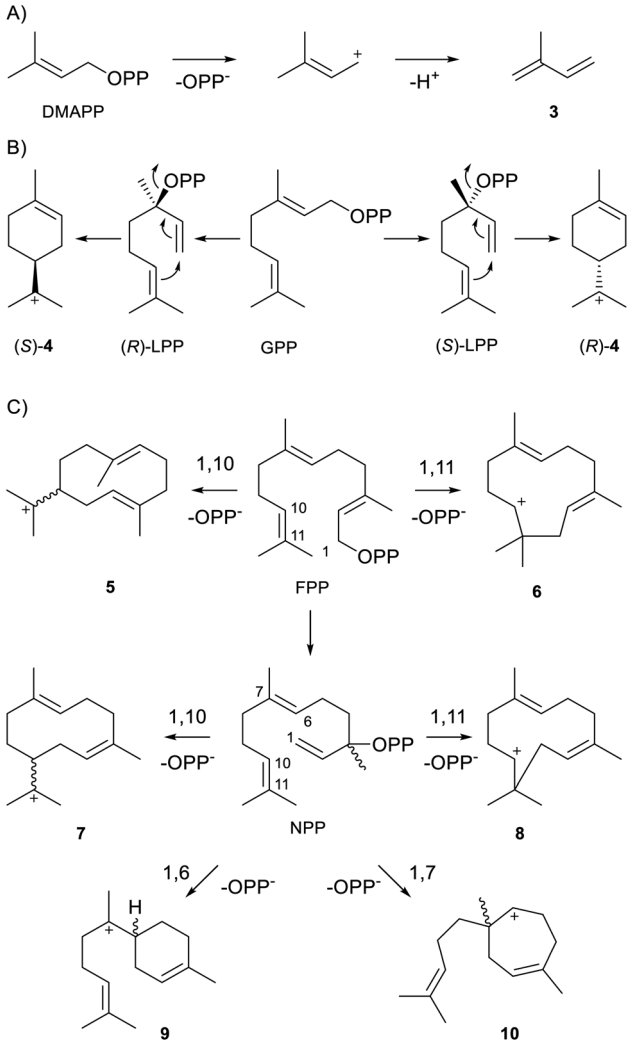
Dana Ulanova defended her PhD thesis on the topic of lincosamide biosynthesis in streptomycetes (under the supervision of Dr Jiri Janata), at the Charles University in Prague, in 2009. The same year she joined the group of Prof. Takuya Nihira at Osaka University as a postdoctoral researcher working on regulation mechanisms of avermectin biosynthesis. In 2011, she was appointed as an assistant professor at Kochi University. Her current research focuses on natural product biosynthesis and ecology in marine microorganisms.



Scheme 1 Terpene biosynthesis. (A) The general principle of terpene biosynthesis proceeds through the sequential events of precursor biosynthesis, followed by cyclisation to unpolar and volatile terpenes, and then functionalisation to yield polar and water-soluble terpenoids. (B) Biosynthesis of oligoprenyl diphosphates from DMAPP and IPP. (C) Formation of squalene and phytoene from FPP and GGPP, the precursors to tri- and tetraterpenes, respectively.

sesquiterpenes, geranylgeranyl diphosphate (GGPP, C20, diterpenes), and geranyl farnesyl diphosphate (GFPP, C25, sesterterpenes). A dimerisation of FPP by squalene synthase leads to the triterpene precursor squalene (**1**, 1,1′-bifarnesyl), requiring a reductive cation quench with NADPH in its formation (Scheme 1C). A similar dimerisation of GGPP, only with terminal deprotonation instead of reduction, results in phytoene (**2**) that is the precursor of tetraterpenes.

TPSs convert the acyclic precursors into terpenes that are structurally often very complex, exhibiting (poly)cyclic skeletons with several stereogenic centers. An exception are hemiterpenes (C_5) that are directly derived from DMAPP that cannot undergo cyclisation reactions. Isoprene is likely the most abundant terpene on earth and is produced by plants in amounts of *ca.* 6 $\times$ 10^{11} kg per year,[32] which is equivalent to *ca.* 100 $\times$ the weight of the Cheops pyramid. Its formation proceeds through



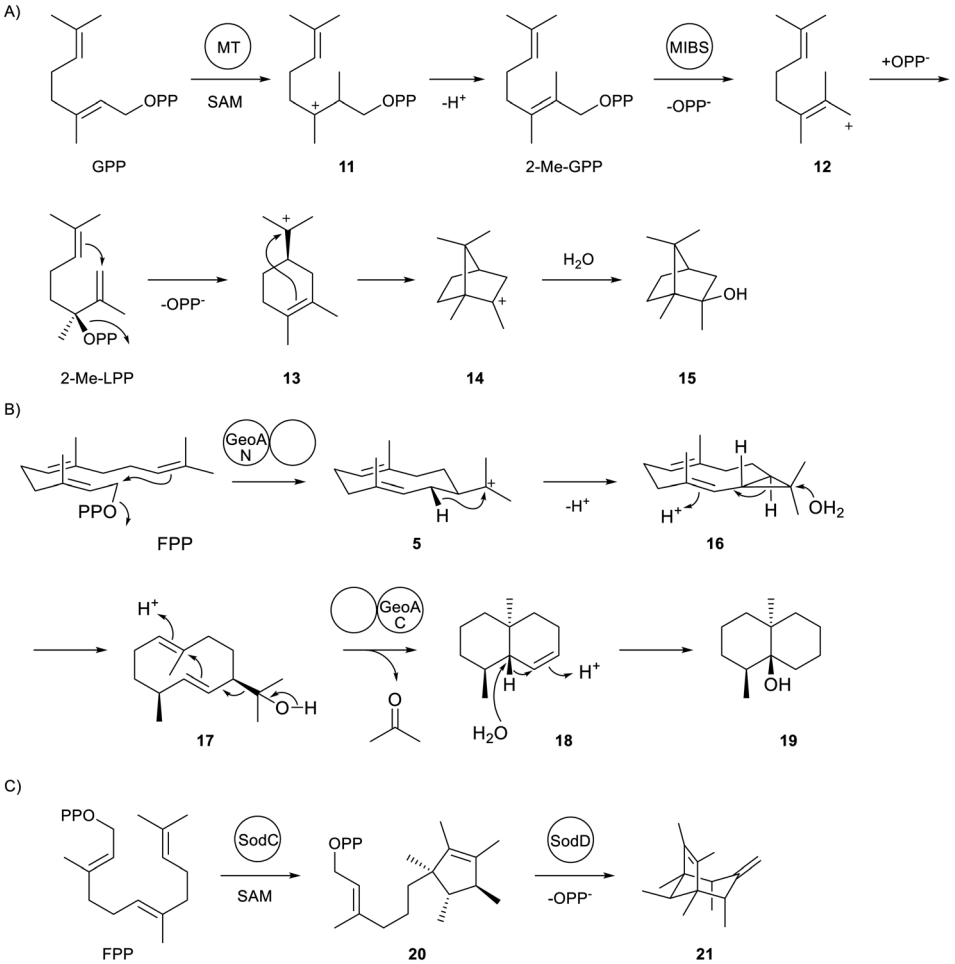
Scheme 2 Terpene biosynthesis by type I TPSs. (A) Formation of isoprene (**3**) from DMAPP, (B) cyclisation of GPP to the (*S*)- or (*R*)-terpinyl cation (**4**), and (C) different initial cyclisation modes of FPP.

abstraction of diphosphate from DMAPP, yielding an allyl cation, and subsequent deprotonation (Scheme 2A). The analogous reaction with IPP is not preferred, because the abstraction of diphosphate leads to a primary instead of an allyl cation. All type I TPSs follow a similar mechanism, *i.e.* they ionise the substrate by diphosphate abstraction, followed by typical carbocation chemistry. This includes cyclisation reactions by intramolecular attack of an olefinic double bond to the cationic center, hydride or proton transfers, and Wagner–Meerwein rearrangements.[12–16]

A specific problem arises for monoterpene biosynthesis because the precursor GPP contains an *E*-configured double bond from C2 to C3, which prevents its instantaneous cyclisation. Therefore, first an isomerisation to either enantiomer of linalyl diphosphate (LPP) is required (Scheme 2B), which upon conformational rearrangement by rotation around the C2–C3 single bond and subsequent abstraction of diphosphate can undergo cyclisation to the (*S*)- or (*R*)-terpinyl cation (**4**).[33] In contrast, for the sesquiterpene precursor a direct 1,10-cyclisation to the (*E*,*E*)-germacradienyl cation (**5**) or a 1,11-cyclisation to the (*E*,*E*)-humulyl cation (**6**) are possible.[34,35] For 1,6- and 1,7-cyclisations to the bisabolyl cation (**9**) or the cycloheptenyl cation (**10**) again first an isomerisation to nerolidyl diphosphate (NPP) is required, allowing rotation around the C2–C3 single bond.[34] NPP can also react by 1,10-cyclisation to the (*Z*,*E*)-germacradienyl cation (**7**) or by 1,11-cyclisation (*Z*,*E*)-humulyl cation (**8**).[35,36] For the larger terpene precursors GGPP and GFPP the number of possible cyclisation modes is further increased, but the general principles remain the same: for 1,6- and 1,7-cyclisations the isomerisation by allylic transposition of diphosphate from C1 to C3 is mandatory, while for all larger rings this step is optional, but can explain the introduction of *Z*-configured double bonds in the biosynthetically last introduced terpene unit.

Besides these regular terpene precursors, a few non-canonical TPSs are known that convert methylated terpene precursors into cyclic terpenes. A well-known example is the biosynthesis of 2-MIB (**15**) for which a mechanistic proposal has been suggested based on isotopic labelling experiments (Scheme 3A).[37] Subsequent characterisation of a small gene cluster composed of genes for an *S*-adenosylmethionine (SAM) dependent methyltransferase (MT) and a TPS (2-MIB synthase, MIBS) and *in vitro* experiments with purified recombinant enzymes confirmed this mechanism.[38,39] The biosynthesis of **15** starts by the methylation of GPP at C2 through transfer of CH_3^+ from SAM to give cation **11**, followed by deprotonation to (*E*)-2-methyl-GPP. The terpene cyclisation first requires isomerisation through cation **12** to 2-methyl-LPP that is subsequently cyclised to the 2-methylterpinyl cation (**13**) and then to the 2-methylbornyl cation (**14**), followed by capture of water to yield **15**. Also, for the biosynthesis of geosmin (**19**, Scheme 3B) the cyclisation mechanism was investigated by feeding experiments with isotopically labelled precursors.[40] The geosmin synthase (GeoA) is a bifunctional enzyme with two domains in which the N-terminal domain catalyses the cyclisation of FPP to the germacradienyl cation (**5**), followed by deprotonation to isolepidozene (**16**).[41] A protonation induced ring opening with attack of water leads to (1(10)*E*,5*E*)-germacradien-11-ol, one of the major side products of GeoA.[42] The C-terminal domain of GeoA catalyses an unprecedented retro-Prins fragmentation of **17** to acetone and the octalin **18**, another side product of geosmin biosynthesis. Its reprotonation is followed by a 1,2-hydride shift and attack of water to yield **19**. Another non-canonical system has been described for the biosynthesis of sodorifen (**21**, Scheme 3C) that also involves an MT (SodC) and a TPS (SodD), but herein the MT not only methylates the precursor FPP, but also catalyses a first cyclisation reaction to presodorifen diphosphate (**20**) that is further converted into **21** by a TPS.[43,44]

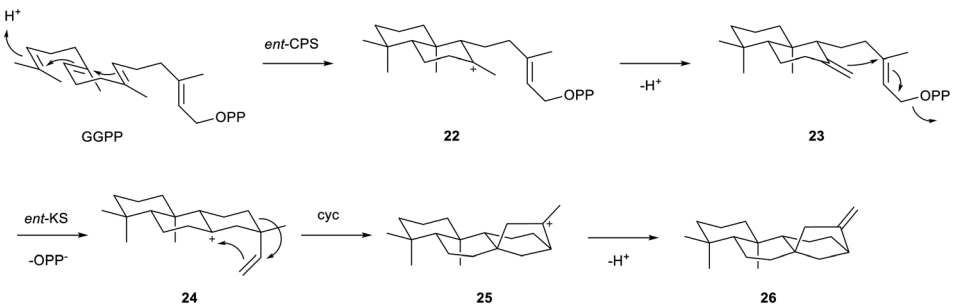
In contrast to the substrate ionisation by diphosphate abstraction as for type I enzymes, type II TPSs induce cyclisation reactions by protonation of the substrate (Scheme 4). One example is the *ent*-copalyl diphosphate synthase (*ent*-CPS) that induces the cyclisation of GGPP to **22** by protonation at C14, followed by deprotonation to *ent*-copalyl diphosphate (**23**). As this product contains an allyl diphosphate group, it can be further converted by the type I TPS *ent*-kaurene synthase (*ent*-



Scheme 3 Terpene biosynthesis by non-canonical type I TPSs. (A) Biosynthesis of 2-MIB (**15**), (B) biosynthesis of geosmin (**19**), and (C) biosynthesis of sodorifen (**21**).

KS) through diphosphate abstraction. Cyclisation to **24** and then with skeletal rearrangement to **25** and final deprotonation lead to *ent*-kaurene (**26**).[45]

While some TPSs apparently only synthesise one specific terpene,[46] others generate many different products.[47] The non-canonical 2-MIB synthase generates several side products whose formation can be understood by premature deprotonation of cationic intermediates along the terpene cyclisation cascade.[48,49] The formation of many products by one enzyme is called product promiscuity, a widespread phenomenon in pathways that produce specialised metabolites. To explain the existence of these conserved pathways, Firn and Jones proposed the screening hypothesis in 1991. This hypothesis states that a large variety of compounds are produced to increase the probability to come across an active compound. Inactive compounds are kept because they might give rise to active compounds in the future.[50] This strategy can be afforded



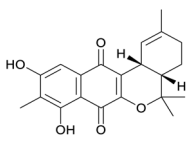
Scheme 4 Terpene biosynthesis by type II TPSs. Cyclisation of GGPP by *ent*-CPS (type II) is followed by further conversion by the *ent*-KS (type I).

because these inactive metabolites are generally produced in very low quantities; therefore, they have a low metabolic cost.[51]

3. Evolution of terpenoid biosynthesis

3.1 Gene transfer in the evolution of terpenoid biosynthesis

The distribution of MVA and MEP pathways, two distinct routes for the biosynthesis of the terpene precursor IPP, is scattered in bacteria and not strongly related to ribosomal RNA based phylogeny. Such distribution can be explained by extensive lateral gene transfer (LGT).[52] LGT plays an important role in the evolution of microbial genomes. Recently, evidence is accumulating of the role of LGT in the evolution of the pathways leading to the biosynthesis of IPP. For example, the enzyme catalyzing the first step of the MVA pathway (HMG-CoA reductase or HMGR) has been shown to be transferred laterally from bacteria to the archaeon *Archaeoglobus fulgidus*.[53] Sequence comparison shows a high similarity between *Archaeoglobus* HMGR and that of *Pseudomonas mevalonii* (class 2 bacterial HMGR). The genome sequence confirmed that *Archaeglobus* does not possess the archaeal or eukaryotic version (class 1) of the HMGR.[52,53] Another example is observed in *Vibrio cholerae*, a bacterial human pathogen that lives in aquatic environments. This bacterium does not have the traditional class 2 bacterial HMGR but a class 1 HMGR including a four amino acid insertion found only in archaeal organisms and not in eukaryotes.[52] *Streptomyces*, a soil Gram-positive multicellular bacterium that is a rich resource of bioactive natural products,[54] can have both IPP biosynthetic pathways. As mentioned above, terpenoids such as menaquinones are produced *via* the MEP pathway while more specialised compounds such as the meroterpenoid antibiotic naphterpin (**27**) are produced using the MVA pathway ("meroterpenoid" meaning of mixed biosynthetic origin, with a terpenoid part).[29,55] The primary role of the MEP pathway suggests the ancestral presence of the pathway while the non-essential MVA pathway could have been acquired at a later stage. The HMGR present in the MVA pathway of many streptomycetes belongs to the class 1 present in eukaryotes and archaea, therefore reinforcing the acquisition of this gene through LGT.[56,57] All these examples are strongly supported by a phylogenetic analysis where the transferred genes clustered with those from organisms which were likely gene donors.[52]



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The high abundance of the MEP pathway first suggested this pathway as the germane pathway in bacteria, with LGT from eukaryotes and archaea explaining the occasional emergence of the MVA pathway.[58] However, more recent phylogenetic studies show that even though archaea and eukaryotes share a conserved MVA pathway, most archaeal species lack the last three enzymes: the phosphomevalonate kinase, the mevalonate-5-decarboxylase, and the isopentenyl diphosphate isomerase (IDI1). Two enzymes, namely isopentenyl phosphate kinase and a non-homologous isopentenyl diphosphate isomerase (IDI2), form the alternative steps of a modified MVA pathway in archaea.[28] A recently discovered superphylum 'Candidate Phyla Radiation' showed a potential MVA pathway that carries enzymes from bacterial and archaeal MVA pathways suggesting that the MVA pathway was present in the last common ancestor of bacteria, and that this pathway was later replaced by the MEP pathway.[28,59]

There is also evidence of inter-kingdom LGT of TPSs. Phylogenetic analysis of bacterial and fungal TPS-coding genes revealed that several fungal TPS genes clustered within the bacterial branch and *vice versa*. Functional analysis of these bacterial-like TPS genes from entomopathogenic fungi confirmed their role in the biosynthesis of several sesquiterpenoids.[60] In another study, genomic analysis of non-Dikarya fungi from the *Basidiobolus* genus, revealed that this genus possess a high number of diverse genes for natural product biosynthesis, which is not typical for other non-Dikarya taxa. Detailed phylogenetic analysis of terpene cyclase (TC) genes revealed that some of them clustered with bacterial TCs. Since one stage of the *Basidiobolus* life cycle happens in animal guts, it was proposed that these genes may have been acquired through LGT with bacteria.[61] LGT of microbial TPSs to eukaryotic organisms is also suggested to be a way of TPS acquisition by red algae and non-seed plants. Phylogenetic and genomic analyses of several red algae species revealed that algal TPSs are more related to microbial-type TPSs rather than to typical plant TPSs.[3] Phylogenetic relatedness together with random distribution in genomes of only a few red algal species indicates that these organisms may obtain TPSs from associated microorganisms. Similarly, microbial-type TPSs were detected in various species of liverworts, mosses, hornworts and other non-seed plants.[62,63]

Another example of evolution in the biosynthesis of terpenes is between TPSs and *trans*-isoprenyl diphosphate synthases (IDSs). These enzymes are non-homologous, however they both possess an "α terpenoid synthase fold" and a trinuclear metal cluster for catalysis. Recently, IDS-like terpene synthases (ILTPSs) were identified in fungi from the genus *Melampsora.* These ILTPSs belong to the family of geranylgeranyl diphosphate synthases (GGDPS) and a phylogenetic analysis suggests that the ILTPSs originate from a GGDPS progenitor in fungi.[64]

The evolution of more complex terpenoids has also been studied, in particular for triterpenoids like hopanoids and tetraterpenoids such as carotenoids. These terpenoids have important functions in microorganisms and will be addressed later in this review. Sterol biosynthesis was thought to be developed by eukaryotes, however, an increasing number of exceptions in bacteria that possess these molecules has raised the question of the origin and evolution of tri- and tetraterpenoids.

Hopanoids and sterols help regulate membrane fluidity in both prokaryotes and eukaryotes. Carotenoids can also provide similar functions as hopanoids and sterols, modulating membrane fluidity and proton permeability.[65,66] The pathways towards the biosynthesis of **1** (triterpenoids) and **2** (carotenoids) are evolutionarily related as isoprenoid-condensing enzymes belonging to the head-to-head connecting *trans*-isoprenyl

diphosphate synthases family are present in the production of **1** as well as in the carotenoid precursor biosynthesis. Squalene (**1**) can be synthesised using two pathways (HpnCDE enzymes and the squalene synthase, Sqs).[67] A recent phylogenetic study shows a closer proximity between the HpnCDE enzymes and those involved in carotenoid production, while the Sqs are more divergent. The distribution and phylogenetic reconstruction points suggest that the bacterial HpnCDE pathway predates the Sqs one.[67]

3.2 Distribution of TPSs in microorganisms

The emergence of sequencing and bioinformatic tools has allowed the study and discovery of microbial TPS sequences. Microbial (bacterial and fungal) type I TPSs conserve metal-binding domains that consist of an aspartate rich motif [(D/N) DXX(D/E) or DDXXXE] (that lies within 80–120 aa of the N-terminus) as well as the NSE triad (closer to the C-terminus). First studies applying hidden Markov models (HMM) using the metal binding domain, indicated that type I TPSs would group into monoterpene, sesquiterpene and diterpene synthases.[39,68] More recent analyses show that the phylogenetic relationship might be more complex than previously thought.[69] The majority of the TPSs analysed were sesquiterpene synthases with three major clades arising corresponding to geosmin synthases, 2-MIB synthases and *epi*-isozizaene synthases.[69,70] TPSs are quite abundant within the *Streptomyces* genus where a similar distribution was found. The majority of TPSs belongs to the sesquiterpene synthase group with geosmin synthase present in 92 out of 93 strains analysed. Other abundant TPSs are 2-MIB synthase and *epi*-isozizaene synthase.[71] In bacteria and fungi, the majority of TPSs produce sesquiterpenoids (type I TPSs), diterpenoids (type I and II TPSs) and triterpenoids (type II TPSs).[7] Despite the abundance of monoterpene compounds found in the headspace of bacteria, hardly any monoterpene synthases have yet been identified, with 1,8-cineole (**28**) synthase from *Streptomyces clavuligerus* as a rare example.[72] Later, an enzyme with the same function, but unrelated amino acid sequence, was described from the endophytic fungus *Hypoxylon* sp.[73] An interesting feature of this enzyme is that it contains an active site asparagine, responsible for water capture and specificity during the biosynthesis of **28**, a mechanism that is used in its plant homologues.[73]

No gene for TPS had been identified in archaeal genomes in previous studies.[67,69] However, squalene/phytoene synthases are widely distributed in archaeal genomes,[67,74,75] in particular, haloarchaea are well-known producers of carotenoids.[76]

In eukaryotes, besides land plants and fungi, TPS genes were also detected in six species of amoebae from the supergroups amoebozoa and excavata. Their phylogenetic analysis demonstrated that amoebal TPSs are more related to fungal TPSs than bacterial ones.[8]

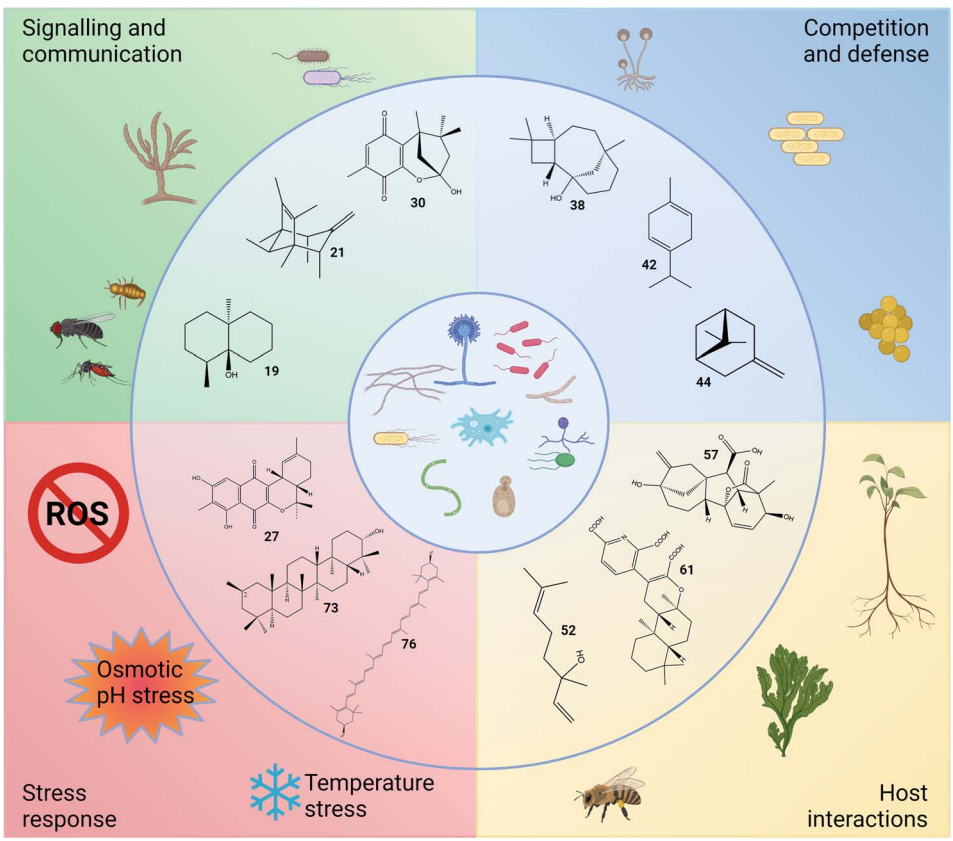


Fig. 1 Overview and examples of the bioecological roles of microbial terpenoids. Created with http://Biorender.com.

4. Ecological roles of microbial terpenoids in aquatic and terrestrial environments

The wide abundance and chemical diversity of terpenoid compounds has motivated researchers to focus on the detection and chemical characterisation of microbial terpenoids. However, the same diversity has recently triggered scientists to also address the biological function of these compounds. Microbial terpenoids are mostly known as infochemicals, important in both intra- and inter-specific communication and interactions. However, these compounds also play an important function in the adaptation of microorganisms to the environment, coping with different biotic and abiotic stresses. Recent examples of the ecological roles of terpenoids in archaea, bacteria, fungi and protists that inhabit diverse aquatic and terrestrial environments are illustrated in Fig. 1.

4.1 Microbial signaling and communication

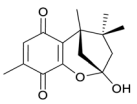
Terpenoid emissions tend to be variable and strongly dependent upon environmental circumstances such as nutrient source and stress exposure reinforcing the interpretation of such molecules in signaling and communication between different organisms.[77,78]

2-MIB (**15**) and **19** are characteristic for their musty to earthy smell and have been known for a long time.[79,80] Geosmin (**19**) is a particularly widespread degraded sesquiterpene[40] produced by many terrestrial and aquatic bacteria including Actinobacteria,[81] Myxobacteria[82] and Cyanobacteria,[70,83] basidiomycete[84] and ascomycete fungi,[85] protists,[42,86] liverworts,[87,88] and arthropods.[89] However, in the last two cases it was not proved whether **19** is produced by the host or its associated microorganisms. As pointed out above, the TPSs for **15** and **19** belong to the two most widely distributed TPSs amongst *Streptomyces.*[38,39,41,71] Both molecules apparently act as intracellular signals and their production correlates to the onset of sporulation in *Streptomyces* species,[90–92] and TPS genes for **15** and **19** are regulated by the sporulation-specific transcription factors BldM and WhiH, respectively.[93] Geosmin may play an important role in the ecology of streptomycetes, as it is recognised not only by microbes, but also by insects. *Drosophila melanogaster* has a very specific sensory mechanism to detect this molecule which allows fruit flies to identify unsuitable feeding and breeding sites due to the presence of harmful microbes.[94] Conversely, mosquitoes sense **19** as a signal for microbial-rich environments suitable for oviposition.[95] Recently, new ecological roles of **15** and **19** were discovered, showing that these molecules attract springtails, which then feed on the mycelia of the streptomycete and subsequently help mediate spore dispersal.[93] Another recent study proposed that **19**, emitted by toxin-producing bacteria, may act as a warning signal for bacteriophagous nematodes, thus reducing the palatability of the producer.[96] Beyond a role in microbial signaling, **19** acts as an inland marker that guides the migration of glass eels to freshwater.[97]

The related compound dehydrogeosmin (**29**) that is produced by Cactaceae, including *Rebutia marsoneri*, *Dolichothele longimamma*, and *Sulcorebutia kruegeri*,[98] has a strongly musty odour, and may play a role as a signal for the attraction of pollinators in the arid environments they live in.[99] It is unknown if **29** is produced by the plant itself or by plant-associated bacteria or fungi.

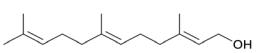
29

Next to constitutively produced terpenoids, microbes can induce terpenoid production as a result of microbial interactions. This is exemplified by the production of the sesquiterpene sodorifen (**21**) by *Serratia plymuthica* when the bacterium is exposed to volatiles produced by the fungus *Fusarium culmorum*.[100] The opposite pattern is also known where the expression of genes responsible for the production of the sesquiterpenoid lagopodin B (**30**) in the fungus *Coprinopsis cinerea* is induced under the presence of bacteria, both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*).[101]



30

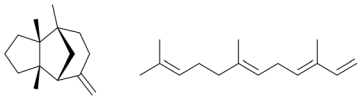
Terpenoids may have different ecological roles depending on the producer microorganism. The sesquiterpene alcohol farnesol (**31**) acts as a quorum sensing molecule in *Candida albicans* and as an antimicrobial compound against *Paracoccidioides brasiliensis*.[102] Quorum sensing is a mechanism that allows microorganisms to detect the presence and density of a population mediated through a small molecule and respond to it. In the polymorphic opportunistically pathogenic fungus *C. albicans*, **31** prevents the fungal transition from yeast to mycelium and disrupts the formation of biofilms.[103,104] However, in *Aspergillus nidulans* the addition of external **31** showed no effect on hyphal morphogenesis, but rather caused morphological changes characteristic of apoptosis,[105] suggesting a role of **31** as mediator of fungal interactions. Interestingly, **31** inhibits the production of the *Pseudomonas* quinolone signal (PQS) and the PQS-controlled virulence factor in another opportunistic human pathogen bacteria, *P. aeruginosa*, indicating the occurrence of interkingdom interactions in the human host.[106]



31

The production of terpenoids by protists has recently attracted attention. Social amoebae like *Dictyostelium discoideum* produce a bouquet of several terpenes like β-barbatene (**32**) and (*E*,*E*)-α-farnesene (**33**) during the mid and late stage of development, suggesting a development-specific role of these compounds.[8] Protists not only produce terpenoids but also sense their prey through these compounds. It is well known that protists prey on bacteria; protists such as *Vermamoeba* and *Tetramitus* sense the

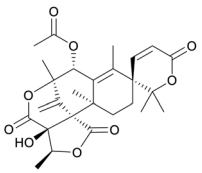
bacteria *Collimonas pratensis* through the production of volatiles, and in particular mono- and sesquiterpenes.[107]



32 33

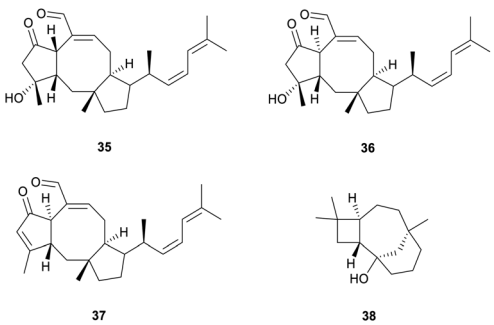
4.2 Microbial competition and defense

Terpenoid production can arise as a microbial mechanism of competition or defense. An example of terpenoid induced production was discovered by the isolation of the meroterpenoid austin (**34**), from the co-culture of two endophytic fungi, *Talaromyces purpurogenus* H4 and *Phanerochaete* sp. H2.[108] This terpenoid was not present in any of the axenic cultures and interestingly, apart from antifungal activity, this molecule has been shown to have also trypanocidal and insecticidal activity, reinforcing the hypothesis of the production of secondary metabolites as a defense mechanism.



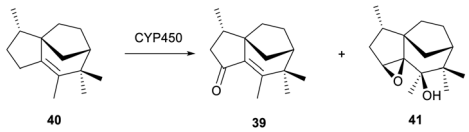
34

Although a lot is still unknown about the suggested competitive benefit of terpenoid production, *in vitro* studies revealed clear inhibitory effects of terpenoids. For example, sesterterpenoids like ophiobolins, *e.g.*, ophiobolin K (**35**), 6-*epi*-ophiobolin K (**36**) and 6-*epi*-ophiobolin G (**37**), produced by marine fungi showed biofilm inhibition in *Mycobacterium* species.[109] The antifungal potential of several *Streptomyces* strains by means of the production of terpenoids could point to a *Streptomyces*-specific defense mechanism when competing for nutrients against fungi within the same niche. Caryolan-1-ol (**38**) is a sesquiterpenoid produced by *Streptomyces* with activity against several different fungi like *Botrytis cinerea*, and *Saccharomyces cerevisiae* probably by inhibiting the endomembrane system.[110]



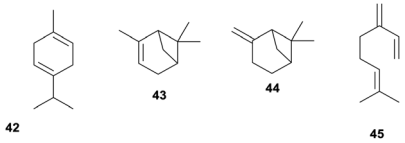
35 36 37 38

Albaflavenone (**39**) is one of the first discovered terpenoid compounds produced by *Streptomyces* sp. with strong antibiotic activity.[111] This compound requires the oxidation of its parent hydrocarbon *epi*-isozizaene (**40**), whose synthase is one of the most widely distributed TPS in *Streptomyces*,[71,112,113] by a cytochrome P450 monooxygenase.[114] A related oxidised metabolite is 4β,5β-epoxy-2-*epi*-zizaan-6β-ol (**41**).[115] Despite their functionalisation, these oxidised terpenoids are still volatile and can be observed in the volatile bouquet of many streptomycetes.[81,116,117]



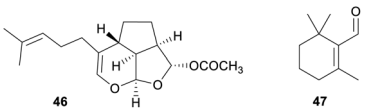
40 39 41

The monoterpenes γ-terpinene (**42**)‡, α-pinene (**43**), β-pinene (**44**) and β-myrcene (**45**) were all detected in the headspace of *Collimonas pratensis* strains Ter91.[118] These monoterpenes were tested individually and as a mixture for antimicrobial activity. β-Pinene exhibited activity against the Gram-positive *Staph. aureus* and against the fungus *Rhizoctonia solani.* Interestingly, a mixture of all four monoterpenes was active against not only the former pathogens but also against Gram-negative *E. coli*.



42 43 44 45

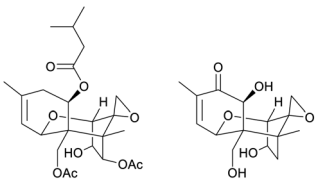
Marine protists belonging to the morphospecies *Euplotes crassus* produce the sesquiterpenoid euplotin C (**46**), which exerts cytotoxic effects on non-producer *Euplotes* strains by altering the cell cycle, ciliary motility and cell shape, resulting in a competitive benefit for *E. crassus*.[119] In Cyanobacteria, the volatile short-chain apocarotenoid β-cyclocitral (**47**) inhibits the competing microalgae and repels grazers such as the planktonic crustacean *Daphnia.*[120]



46 47

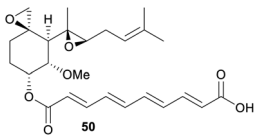
An extended defense mechanism amongst fungi is the production of toxins. Some toxins belong to terpenoids, such as the trichothecenes. These molecules are sesquiterpene-based mycotoxins that inhibit protein synthesis.[121] One of the best known examples is the T-2 toxin (**48**) produced mostly by plant pathogens such as *Fusarium*, *Myrothecium* and *Trichooderma* among others.[121] Another example is deoxynivalenol (**49**), a type B trichothecene, produced mainly by *Fusarium gramineum*. This toxin has been thoroughly studied due to its harmful effects such as vomit and diarrhea in humans by the ingestion of contaminated grains.[122]

‡ Here and later, if not mentioned, the absolute configuration was not reported.



48 49

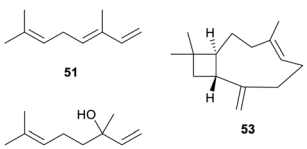
Fumagillin (**50**) is a toxin produced by *Aspergillus fumigatus*, with a characteristic structure of a rearranged and highly oxygenated sesquiterpenoid and a polyketide-derived tetraenoic diacid.[123] This toxin showed an amoebicidal effect against *Entamoeba histolytica*[124] suggesting a possible role of fungal virulence in the defense against amoeboid predators.[125]



50

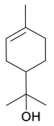
4.3 Host–microbe interactions

Terpenoids are best known as plant metabolites, but these compounds are also produced by many plant-associated microbes and play an important role in plant–microbe chemical interactions.[126,127] Various microbial terpenoids have plant growth-promoting activity or provide protection against abiotic or biotic stresses, acting on pests and pathogens that pose a threat to plant health (reviewed in ref. 126 and 128). Infection of plants by microbial pathogens can trigger the emission of terpenoids in several plant species. For example, upon infection by *Fusarium* spp. maize plants showed a rapid emission of pathogen-suppressing sesquiterpenes.[129] The terpenoid production of potato plants was affected by an inoculation with *Phytophthora infestans.*[130] Triggering terpenoid emission following a pathogen attack is a response in which the plant-associated microbiome likely plays a major role. Similarly, plant terpenoids (such as **28**, (*E*)-β-ocimene (**51**), linalool (**52**), (*E*)-β-caryophyllene (**53**) and many others) play important roles in plant–insect, plant–pathogen and plant–plant interactions.[131–133] Since both plants and microbes produce terpenoids, the true producer (plant, microbe, or both) often remains to be elucidated.



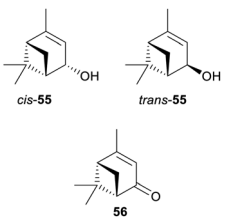
51 53 52

Floral microbiota can significantly influence plant emissions, *e.g.*, removing the floral microbiota of *Sambucus nigra* plants affected both the quality and quantity of terpenoid emissions.[134] Floral nectar is a rich source of sugars and commonly colonised by yeasts. Yeasts produce a blend of volatiles including terpenoids such as **52**, α-terpineol (**54**), **45**, or **33** (ref. 135) that attract insects which in turn feed on the sweet nectar while serving as pollinators for the plant[136] and dispersal vectors for yeasts.[137–139] The shared volatile terpenome profile between yeast and plants suggests an important role of the compounds in plant–insect–yeast interactions.[135]



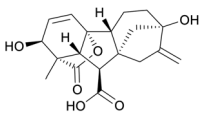
54

Cis/*trans*-verbenols (**55**) and verbenone (**56**), (anti-)aggregation pheromones of bark beetles, are produced by conversion of **43** to **55**, either by the beetles themselves, or by their microbial symbionts.[140,141] Then, **55** can be converted to **56** by associated bacteria, yeast and fungi.[142–144] Interestingly, *cis*-**55** has a higher antibacterial activity than **56**, thus its conversion seem to be beneficial for both beetles and their bacterial symbionts.[142]



cis-**55** *trans*-**55** **56**

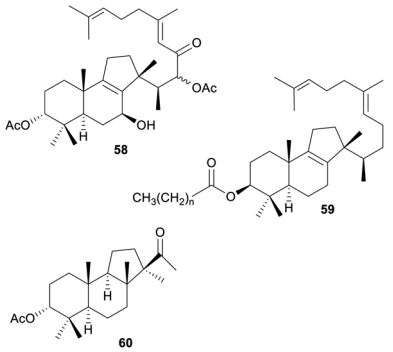
Gibberellins (GAs), *e.g.* gibberellic acid (**57**), are a large family of tetracyclic diterpenoid carboxylic acids that are biosynthetically derived from **26**.[145] These plant hormones are required for many developmental processes such as seed germination, organ elongation, trichome development, flower, seed and fruit development.[146] GAs are also produced by fungi with an effect on plants. Production of high amounts of GAs by *Fusarium fujikuroi* isolated from rice correlates to the appearance of 'bakanae' (Japanese for foolish seedling) disease characteristic of yellow and elongated rice seedlings.[147,148] During symbiosis between the plant *Eustoma grandiflorum* with arbuscular mycorrhizae, exogenous GAs promote the fungal entry and colonisation as well as arbuscule formation in the root cortex.[149] GAs also play a role in the interaction of fungi with different hosts. In the human fungal pathogen *Cryptococcus neoformans*, an increased production of GAs was observed as a response to testosterone which allowed the fungus to also increase its melanin production.[150] Melanin plays an important function in this fungus as it enables it to avoid phagocytosis,[151] and even when phagocytosed, melanin protects *C. neoformans* from the oxidizing agents produced by the macrophages allowing its survival and replication.[152]



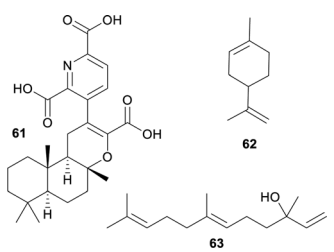
57

In belowground plant–microbe interactions, microbial terpene biosynthetic genes were shown to be enriched in the

endophytic microbiome of plant roots under attack by the fungal pathogen *Rhizoctonia solani*.[153] However, little is currently known of the ecological conditions under which the genes for microbial terpene biosynthetic enzymes are expressed, or of their biological functions in microbe–plant interactions. On the other hand, plant triterpenoids thalianin (**58**), thalianyl fatty acid esters (**59**), and arabidin (**60**) released from roots of *Arabidopsis thaliana* were shown to modulate microbiome assembly and serve as carbon sources for some bacteria.[154]



Microbial terpenoids are also known to promote host development and health in the aquatic environment. The tripartite chemical interactions of a green alga, *Ulva*, with the bacteria *Roseovarius* sp. (Roseobacter clade, Rhodobacteraceae) and *Maribacter* sp. are essential for host growth, cell differentiation and rhizoid formation.[155,156] *Ulva* releases dimethylsulfoniopropionate, which attracts *Roseovarius* sp. and other bacteria.[157] *Roseovarius* sp. produce unknown morphogenetic compounds, which act similar to cytokinin and stimulate macroalgal cell division and growth.[155] A third member of this interaction network is *Maribacte*r sp., which produces the meroterpenoid thallusin (**61**). This compound was originally isolated from the marine epiphytic bacterium associated with another green alga, *Monostroma oxyspermum* and induces rhizoid and cell wall formation.[156,158] Similar morphogenetic activities were detected in other bacterial species associated with different *Ulva* species. However, the chemical identity of the morphogens remains unknown.[159,160] The terpenoids limonene (**62**), nerolidol (**63**) and **31** are produced by *Pseudooceanicola algae*, a bacterial species that grows on the surface of the brown alga *Fucus spiralis*, and proposed to have a role in algal surface defense and bacterial symbiont interaction.[161]

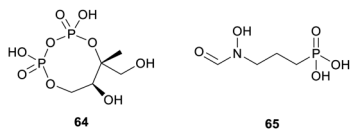


4.4 Microbial stress response

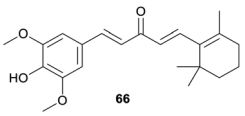
In nature, microorganisms are subject to constantly fluctuating environmental conditions and diverse abiotic and biotic stresses. Stressful conditions can severely impact growth and survival; thus, microorganisms rely on various adaptation mechanisms allowing them to adjust to a specific situation. Like in plants, terpenoids can play a role in the adaptation of microorganisms to common stresses, such as oxidative, nitrosative, temperature, osmotic, pH and nutrient stress.

4.4.1 Oxidative and nitrosative stress. One of the most common stressors encountered by bacteria is oxidative stress, which is caused by reactive oxygen species (ROS). Under normal conditions, these molecules are rapidly degraded by enzymes such as superoxide dismutases, catalases and peroxidases. Exposure of cells to ROS causes damage to DNA, proteins and membrane lipids, and may even lead to cell death.[162] Nitrosative stress is similar to oxidative stress, but is caused by an increase in reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($OONO^-$).[163] RNS are by-products of anaerobic denitrification in bacteria which are usually kept at low concentrations.[164,165] Oxidative and nitrosative stress can have a suppressive effect on the MEP pathway. More specifically, ROS and nitric oxide (NO) inhibit the final enzymes (IspG and IspH) of the pathway that both contain an iron sulfur cluster that is sensitive to oxidation. The inhibition of these enzymes leads to substantial accumulation of the IspG substrate 2-*C*-methyl-D-erythritol-2,4-cyclopyrophosphate (MEcPP, **64**). MEcPP (**64**) itself is an effective antioxidant and has been suggested to capture ROSs to protect IspG and IspH, in order to recover the pathway.[166] The antioxidant activity of **64** has also proven to be effective in preventing DNA damage.[166]

A recent study on the effects of sub-inhibitory fosmidomycin (**65**) treatment in *Salmonella enterica* further highlights the relevance of the MEP pathway in responding to oxidative stress.[167] Fosmidomycin (**65**) is an inhibitor of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), the enzyme which catalyses the first committed step of the MEP pathway. The addition of **65** significantly increases the sensitivity of *Salmonella enterica* to oxidative stress due to the disruption of the MEP pathway. In comparison, treatments with kanamycin and tetracycline antibiotics that do not act upon the MEP pathway, only elicit a relatively small response to oxidative stress.[167]

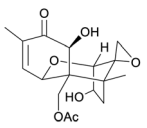


Apart from the role of the terpene biosynthetic pathway in the response to oxidative and nitrosative stress, many terpenoids show antioxidant potential *in vitro* and likely serve as protectants against oxidative stress. Among terpenoid antioxidants are the monoterpene **62** and the meroterpenoids **27** and nostocionone (**66**).[168–170] To confirm their function as oxidative stress protectants in bacteria, these compounds should be further investigated *in vivo*.



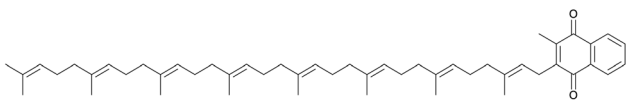
66

The role of terpenoids in oxidative stress response is also known for other microorganisms. A common response to oxidative stress in fungi is induced mycotoxin production. The plant pathogen *Fusarium graminearum* responds to oxidative bursts encountered upon infection with increased production of the trichothecene sesquiterpenoids **49** and 15-acetyldeoxynivalenol (**67**). This stress reaction is regulated by Fgap1, a homologue to the oxidative stress responsive transcription factor Yap1 in yeast.[171]



67

Menaquinones, *e.g.*, menaquinone-8 (**68**), which contain oligoprenyl side chains of different lengths, are a major constituent of haloarchaea membranes and are suggested to protect cells against extreme oxidative stress by functioning as permeability barriers.[172]



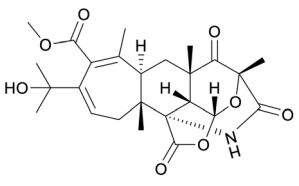
68

4.4.2 Temperature, osmotic, pH and metal stress. Elevated temperatures induce misfolding of proteins, triggering the heat shock response (HSR). Low temperatures reduce enzyme activity, decrease membrane fluidity and lower efficiency of transcription, translation and protein folding. Increasing the fluidity of the cell membrane by modifying its composition also increases growth at low temperatures. Modulation of membrane fluidity seems to be an important function of terpenes in cold stress. In *Listeria monocytogenes* high isoprenoid quinone concentrations cause fluidisation of the membrane and support growth at low temperatures,[173] while in *Escherichia coli*, a mutation in the gene *ispA*, encoding farnesyl diphosphate synthase (FPS) improves growth at low temperatures.[174]

Osmotic stress is caused by changes in environmental solute concentration and osmotic pressure. Microorganisms can adapt to osmotic stress by altering intracellular solute concentrations, either of inorganic salts or of organic compounds called osmolytes, which are often zwitterionic. Bacteria that are adapted to living in hypersaline conditions sometimes produce biosurfactants, which reduce surface and interfacial tension. Biosurfactants produced by the halophilic bacterium *Planococcus maritimus* are believed to be synthesised from terpenes.[175] Like oxidative stress, osmotic stress can trigger an increased production of terpenoid mycotoxins in fungi. In *Fusarium graminearum*, the production of the trichothecene **49** is regulated by the response regulator FgRrg-1 that is involved in osmotic stress response.[176]

Acidification of cells can cause a lowered enzyme activity, unfolding of proteins, membrane damage and DNA damage. The effect of high pH on bacterial cells is less well-known, however, alkaline conditions can also elicit a strong stress response. Adaptation to acid stress in the lactic acid bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus* includes repression of the MVA pathway, the singular route for terpene biosynthesis in this genus. Its repression favours the biosynthesis of fatty acids in order to change membrane composition and enhance protection against the acidic environment.[177] Environmental pH may influence the activity of enzymes involved in terpene biosynthesis. The catalytic mechanisms of many TPSs depend on acid–base reactions.[178]

The increased concentrations of trace metal ions can be toxic for living cells. The marine fungus *Aspergillus* sp. WU 243 found in the digestive gland of the hydrothermal vent crab *Xenograpsus testudinatus* produces the polyketide terpenoid aspergstressin (**69**), a molecule of unknown absolute configuration, only when exposed to cobalt stress, although its functioning in this context is not yet known.[179]



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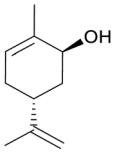
4.4.3 Nutrient deprivation. Nutrient stress is caused by a depletion of essential compounds such as carbon sources, iron and phosphate. In (facultatively) anaerobic bacteria containing both the MEP pathway and the MVA pathway, the available carbon source determines which pathway is used.[180] Some bacteria utilise plant-derived terpenoids as a carbon source. The monoterpene **62** is commonly used as a sole source of carbon and energy.[181] A *Pseudomonas* sp. strain has been described that can convert **43** and **44** into *p*-menthene derivatives including **62**, products which can be used as sole carbon sources.[182] Bacterial utilization of **19** (ref. 183) and various monoterpenoids, including stereoselective degradation of **62** and carveol (**70**), have also been described.[184,185] In the case of **70**, (4*R*,6*S*)-**70** was converted fastest in enzyme reactions.[184]



70

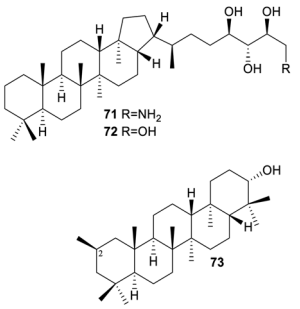
Hopanoids and carotenoids play a role in all the types of stress described above and have been extensively researched. The findings are summarised next.

4.4.4 Hopanoids in the bacterial stress response. Hopanoids are planar polycyclic triterpenoids with structural similarity to sterols, though containing five carbon rings instead of four. They are mainly produced by bacteria, although some are produced by plants and lichens. Hopanoids are produced from squalene by enzymes called squalene–hopene cyclases (SHCs) and can intercalate into membranes, thereby decreasing their fluidity while simultaneously increasing their rigidity. Hopanoids cannot fully compensate for sterol deficiency, indicating that the two classes are functionally distinct.[186] Hopanoid production is also associated with nitrogen fixation and plant–bacteria interactions.[187] A recent transcriptomics study links hopanoids to chemotaxis and membrane transport in *Rhodopseudomonas palustris*.[188]

Deletion of the hopanoid biosynthetic genes in *Rhodopseudomonas palustris* and *Methylobacterium extorquens* impairs growth when exposed to high and low pH, bile salts and antibiotics.[189,190] There is evidence that hopanoids help to protect root-associated bacteria against external stresses. Hopanoid deficiency in *Bradyrhizobium*, a nitrogen-fixing symbiont of legumes, increases its sensitivity to oxidative stress, osmotic stress, detergent, and low pH. Members of the human and plant pathogenic genus *Burkholderia* also rely on hopanoids for stress protection. In these bacteria, hopanoids increase resistance to low pH, detergent and various antibiotics including polymyxin B, erythromycin, chloramphenicol and colistin.[191,192]

In some species, hopanoids are only advantageous for specialised, often stress-related cell types. In *Streptomyces coelicolor*, hopanoid production is limited to the developmental growth phase.[193] Hopanoids are produced in so-called *whi* mutants, which form aerial mycelium but fail to produce spores. Hopanoids are also not produced by many of the so-called *bld* mutants, which only grow vegetatively and cannot form an aerial mycelium. They are likely produced in response to osmotic stress encountered upon aerial growth, which they alleviate by diminishing water diffusion across the cell membrane.[193] In *Nostoc punctiforme*, hopanoids are not essential for vegetative cells but they are required for stress tolerance in akinetes, a resting survival cell type that appears under harsh conditions like extreme cold or drought.[194]

In *Bradyrhizobium diazoefficiens* different hopanoid classes are required during its free-living state as opposed to its symbiosis state.[195] In this bacterium, C35 hopanoids such as hopanoid-derived aminotriol (**71**) and bacteriohopanetetrol (**72**) are necessary for microaerobic growth and they are required for symbiosis with its plant host *Aeschynomene afraspera*.[195] In contrast, 2-methyl-hopanoids such as **73** are not needed for symbiosis, but promote growth under microaerobic and acidic conditions, which suggests that methylation of hopanoids differentially affects their function, although it is not yet clear how.[195]

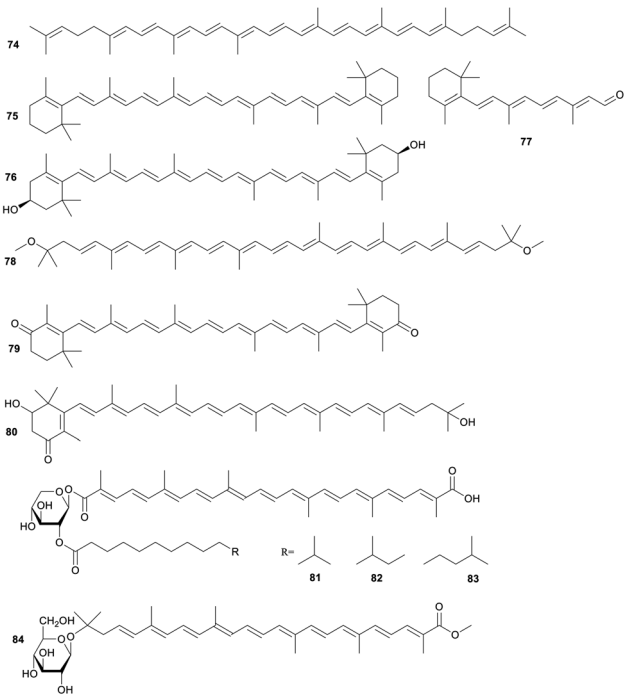


In *N. punctiforme*, 2-methyl-hopanoids are required for pH and osmotic stress tolerance, but not for akinete formation.[196] Interestingly, in *Rhodopseudomonas palustris*, the gene for C2 hopanoid methylase (*hpnP*) is regulated by the general stress response factor EcfG. Upregulation of *hpnP* is associated with various stresses, including high temperature, acidic and alkaline conditions and osmotic stress induced by nonionic solutes. SHCs, which catalyse the first step of hopanoid biosynthesis, do not appear to be regulated by EcfG. This means that EcfG-dependent regulation is likely specific to 2-methyl-hopanoids and not related to hopanoid production in general.[197]

4.4.5 Carotenoids and microbial stress response. Carotenoids are tetraterpenoids widely produced by plants and algae, and are present in the membranes of both photosynthetic and non-photosynthetic bacteria. They can be broadly categorised into two groups, the oxygen-containing xanthophylls and the unoxygenated carotenes. Phytoene (**2**), the first intermediate of carotenoid biosynthesis, is modified by desaturases, isomerases and cyclases to yield various carotenoids, such as lycopene (**74**) and β-carotene (**75**). Further modification of these derivatives, such as ketolation, hydroxylation, glycosylation or oxidative cleavage, leads to the formation of numerous other carotenoids and apocarotenoids, for example zeaxanthin (**76**) and retinal (**77**).[198,199] The majority of carotenoids have a C40 structure, however C30-, C45- and C50-carotenoids can also occur.[200]

Carotenoids are synthesised by all photosynthetic bacteria, where they enhance light harvesting and electron transfer during photosynthesis, offer protection against photodamage and serve important roles in the assembly and stabilisation of the photosynthetic machinery.[201] Moreover, many carotenoids have strong antioxidant effects and are crucial for oxidative stress resistance. For example, the photosynthetic genus *Bradyrhizobium* contains two distinct carotenoid biosynthesis clusters (*crt*); one involved in photosynthesis and light-regulated producing spirilloxanthin (**78**), and the other one involved in the oxidative stress response by the synthesis of canthaxanthin (**79**).[202] The antioxidant function of carotenoids is derived from their conjugated double bond system which permits quenching of singlet oxygen. Structural variety among carotenoids allows for protection against various other ROS. Antioxidant activity is further influenced by their concentration, orientation within the membrane, interaction with other antioxidants and the partial pressure of oxygen.[203,204] An exceptionally strong antioxidant is deinoxanthin (**80**), a unique carotenoid produced by the extremophilic *Deinococcus radiodurans*.[205,206] Novel acyclic carotenoids with a C_{30} aglycone, diapolycopenedioc acid xylosylesters (**81–83**) and methyl 5-

glucosyl-5,6-dihydro-apo-4,4′-lycopenoate (**84**) potent antioxidant activity have been isolated from marine bacteria such as the Gram-negative *Rubritalea squalenifasciens* and the Gram-positive *Planococcus maritimus*. These bacteria might produce carotenoids to protect themselves from activated oxygen produced by sunlight.[207]

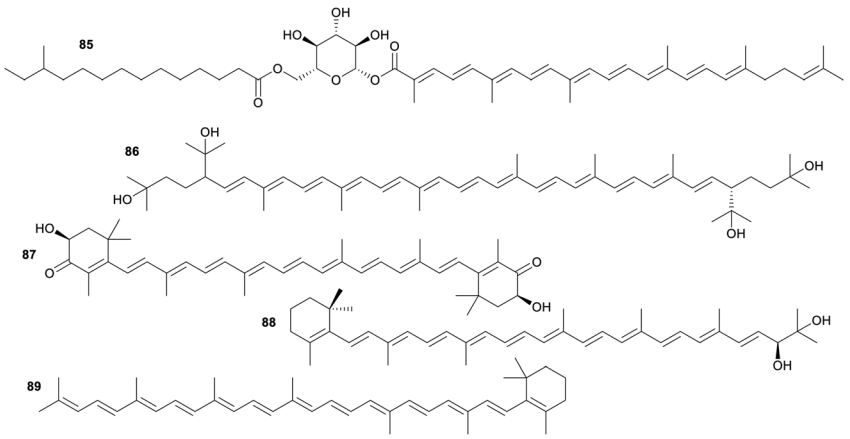


Besides ROS scavenging, carotenoids also confer protection against oxidative damage through their rigidifying effect on membranes, which limits oxygen penetration into the membrane.[65] A decrease in membrane fluidity may also have a positive effect on the response to other stresses, such as cold stress. In antarctic heterotrophic bacteria, carotenoid pigmentation correlates with an increased resistance to freeze–thaw stress.[208] Additionally, the psychrotrophic bacterium *Arthrobacter agilis* synthesises more carotenoids when grown at low temperatures compared to high-temperature cultures.[209] Carotenoids are also suggested to play a role in the ethanol tolerance of the wine bacterium *Oenococcus oeni*, which highly expresses GGDPS under ethanol stress.[210] The relation between carotenoids and osmotic stress is less clear. While high salinity content in wastewater enhances carotenoid production in a photosynthetic *Rhodopseudomonas* strain,[211] production decreases under salinity stress in *A. agilis*.[209] Metabolic engineering and heterologous expression of carotenoid biosynthesis genes in *B. subtilis* and *Lactococcus lactis* showed that the production of carotenoids caused increased resistance to various stresses such as oxidative stress and acidic stress.[212,213]

Carotenoids play an important role in the virulence of several pathogenic bacteria. The golden carotenoid pigments of the human opportunistic pathogen *Staph. aureus* offer protection against oxidant-based attack by neutrophils.[214,215] This is at least partly mediated by the ROS scavenging ability of the carotenoid staphyloxanthin (**85**).[216] In so-called group B *Streptococcus*, streptococci group which causes pneumonia and meningitis in neonates,[217] carotenoids protect against oxidative burst killing mechanisms of phagocytes.[218]

Likewise, carotenoids are important stress response compounds in other microorganisms. In haloarchaea, carotenoid bacterioruberin (**86**) acts as a potent radical scavenger, controls cell membrane rigidity and protects the cell against extreme environmental conditions.[76,219] In fungi, which produce a range of carotenoids, these compounds play a role in protection against ROS and damaging UV light.[220] For example, inducing oxidative stress in the fungus *Blakeslea trispora*, the main producer of carotenoids for industrial use, significantly enhances carotene production.[221,222]

Treatment of antioxidant-deficient *S. cerevisiae* strains with the xanthophyll carotenoid astaxanthin (**87**) reduces ROS levels and prevents oxidative stress induced cell death, proving its widespread importance as an antioxidant.[223] Astaxanthin (**87**) production does not naturally occur in *S. cerevisae*, but is found in diverse microorganisms such as the yeast *Xanthophyllomyces dendrorhous* and the bacteria *Paracoccus* sp.[224] Red yeasts *Phaffia rhodozyma* and *Dioszegia* sp. overproduced **87** and plectaniaxanthin (**88**), respectively, when they were subjected to oxidative stress.[225,226]



Another red yeast *Sporidiobolus pararoseus* increased the production of torulene (**89**) under salt stress induced by high NaCl treatment, which can also induce oxidative stress.[227,228]

5. Terpenoid analysis and application

5.1 The collection and analysis of terpenoid compounds

Many terpenoid compounds are volatile and one of the many challenges when working with volatile compounds is the correct trapping of them, as these molecules can easily diffuse and be lost or even further, influence the behavior of neighbouring organisms. A few different methods have been developed such as using a Petri dish designed to hold a stainless steel trap containing adsorbents like Tenax® or Carbopack B.[229,230] To mimic more natural systems, and analyze diffusion of volatile compounds in soil, a pot-in-jar system and an olfactometer-choice assay have been recently developed.[231,232] To trap VOCs directly from the natural environment, silicon tubes, *e.g.*, polydimethylsiloxane (PDMS) tubes can be used.[233] The trapped volatile compounds can be further analyzed using gas chromatography coupled to mass spectrometry.[234] The obtained mass spectra can be identified by comparison with databases and mass spectral libraries such as the NIST library (http://webbook.nist.gov/chemistry/), the Pherobase (http://www.pherobase.com/), MassFinder (http://massfinder.com/), the Dictionary of Natural Products[1] or in-house databases. Platforms for metabolomics data analysis such as MetaboAnalyst allow the processing of raw data into a comprehensive and many times user-friendly statistical and functional (meta) analysis of molecules.[235] The Global Natural Products Social Molecular Networking (GNPS), a recently established community-driven MS data sharing platform, provides tools for high-throughput identification and dereplication of mass spectral data using datasets across a range of model organisms and systems.[236] The workflow was originally developed for LC-MS data; however, a novel machine learning approach has enabled processing GC-MS data and performs molecular networking within the GNPS platform.[237] Techniques for the interpretation of MS data as well as GC-MS based structure elucidation were recently reviewed elsewhere.[234] Several other natural product databases have been recently released such as the MIBiG repository which holds experimentally characterised biosynthetic gene clusters (BGCs).[238] Knowledge on the BGCs of TPSs allows the use of online tools such as antiSMASH to locate TPSs in the genomes of sequenced microbes.[239] This data can be further analysed using algorithms such as BiGSCAPE and CORASON that enable the exploration of big datasets from diverse organisms based on sequence similarity.[240] The big advances in bioinformatic tools and the analysis of BGCs have been key to the study of the genomic basis of natural product biosynthesis. However, the structure of the terpenoids that are specified by the biosynthetic genes cannot yet be predicted from the genomic data alone. A general workflow for the mining, identification, production and exploitation of volatile terpenoids is presented in Fig. 2.

5.2 Applications in medicine, food and agriculture

As discussed above, terpenoids have highly diverse bio-ecological roles. Like many other secondary metabolites, terpenoids have many biological activities including antimicrobial, anti-oxidative, anti-inflammatory and anti-cancer, and this makes them potentially attractive for application in human health, as food protectant and in agriculture. A couple of examples of plant-derived terpenoids being produced by pharmaceutical industries and generating multibillion dollar proceeds are the anti-cancer diterpenoid paclitaxel (Taxol®, **90**) and the anti-malarial sesquiterpene lactone artemisinin (**91**).[241] Interestingly, paclitaxel was originally discovered from the Pacific yew,[242] but later was also isolated from its fungal endophyte.[243] However, the independent biosynthesis of this and related compounds by endophytic fungi is still disputable.[244]

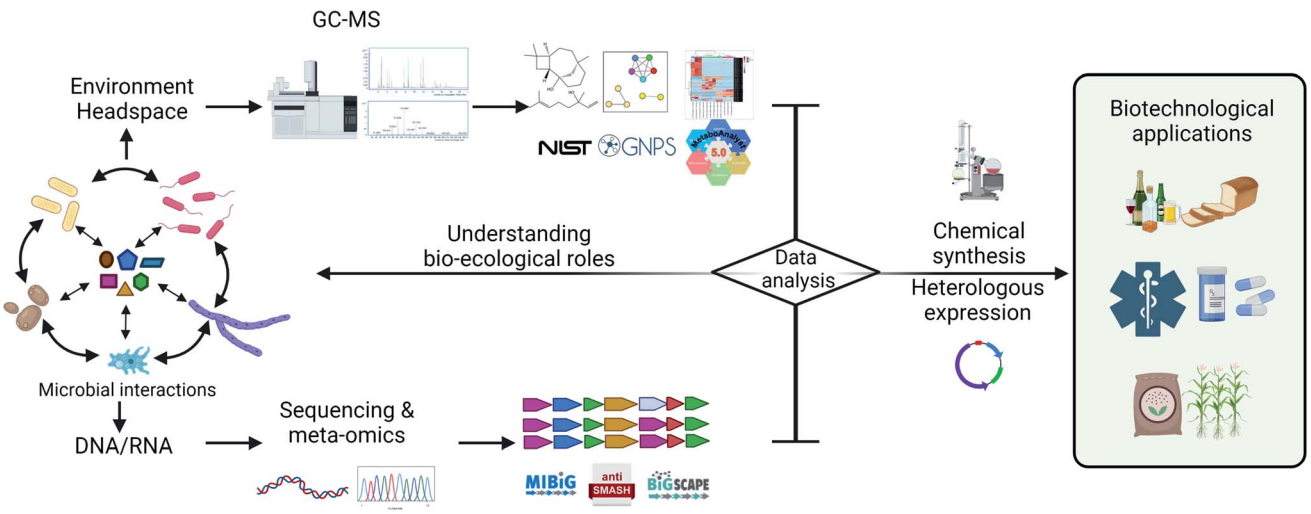


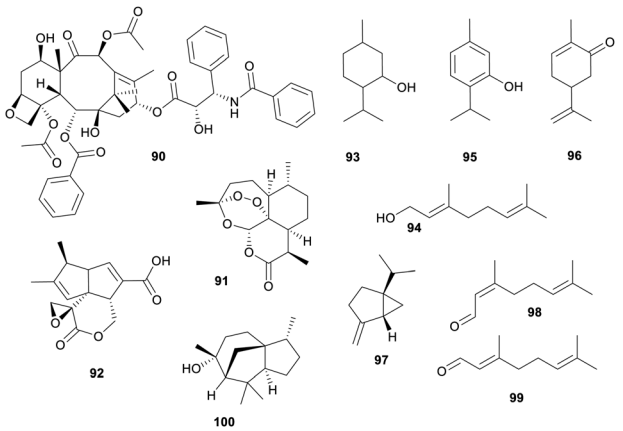
Fig. 2 Workflow showing key steps in the analysis of volatile terpenoids and their applications in biotechnology. Created with http://Biorender.com.

The antimicrobial activity of terpenoids has been studied extensively.[245] Examples date from 1957 when the antimicrobial sesquiterpenoid pentalenolactone (**92**) was discovered from *Streptomyces roseogriseus* with antibacterial activity against Gram-positive and Gram-negative bacteria.[246] Monoterpenes such as **42**, **43**, **44** and **45** are antibacterial agents with activity against *Staph. aureus* and *E. coli*.[118] In case of **43** and **44**, the bioactivity depends on compound stereochemistry – (+)-**43** and (+)-**44** had antimicrobial activity against all tested fungi and bacteria, while no activity were detected when applying their enantiomers.[247]

Another possible clinical application of terpenoids can be influencing of quorum sensing activity of pathogens. The monoterpene (*R*)-**62** exerts an anti-quorum sensing activity for *Escherichia coli* influencing its biofilm formation, curli expression, swimming and swarming motility when the compound is present in a nanoemulsion.[248] Another study reported that **15** inhibits the sensor for *N*-3-oxohexanoylhomoserine lactone (quorum-sensing signaling molecule) in *in vitro* assays.[249]

The antimicrobial activity of terpenoids extends beyond the sole effect of the single compounds. Menthol (**93**) can be effective against *Staph. aureus* and *Bacillus cereus* or only *B. cereus* when applied in combination with geraniol (**94**) or thymol (**95**), respectively.[250] Similar synergistic associations were observed for terpenoid treatments in combination with known 'canonical' antibiotics. Treatment of antibiotic-resistant *Staph. aureus* and *E. coli* with penicillin was effective in combination with terpenoids carvone (**96**) and **95**, respectively.[251] Limonene (**62**), sabinene (**97**) and **43** among others, were shown to enhance activity of anti-tuberculosis drugs such as ethambutol and rifampicin.[252] Enantiomers (+)-**43**/**44** exhibited synergistic activity with ciprofloxacin against methicillin-resistant *Staph. aureus*.[247] However, (−)-**43**, inactive when applied on its own, increased the susceptibility of resistant *Campylobacter jejuni* to ciprofloxacin, erythromycin and triclosan by modulating membrane integrity and inhibiting antimicrobial efflux.[253] Inhibition of biofilm formation, disruption of cell membrane integrity and synergistic activity with other antimicrobial compounds was also reported for **31** when tested against *Staph. aureus*.[254]

The application of terpenoids to improve human health and lifestyle extends to their use in food and cosmetics. Sachets containing phenylpropanoid eugenol and citral (mixture of terpenoid isomers neral (**98**) and geranial (**99**)) allow prolonging the shelf life of bread without influencing the odour of the food.[255] Flavour and fragrances is a big market for these types of compounds. The global market for flavour and fragrance ingredients was valued at $1.4 trillion is 2019 and is expected to rise to $1.8 trillion in 2024.[256] Many of the aromas used in food and fragrances are blends of terpenoids; lemon-lime sodas are given the flavour and aroma with a mixture of **62**, **52** and **28** among others,[257] while parfume's key active ingredients are blends of terpenoids such as **94** and cedrol (**100**) characteristic in the smell of roses and cedar wood, respectively.[258]



Since many terpenoids were originally isolated from plants, limited supply or the requirement for chemical synthesis often limited their application. However, we now know that many plant-derived terpenoids can also be produced by microorganisms, which opens the door for more ecology-friendly and sustainable production. Furthermore, the advanced knowledge on biosynthesis and chemistry of terpenoids can be applied for novel biotechnological approaches for terpenoid synthesis in microorganisms.[259–261] In addition, a modular *in vitro* platform for the production of mono- and sesquiterpenoids from CO_2 was recently designed by combining acetyl-CoA (terpenoid building block) and terpene biosynthetic pathways.[262]

Despite the abundant use of synthetic fertilisers and pesticides, more than one third of crop yield is currently lost due to abiotic and biotic stress factors. At present, one major challenge facing agriculture is to secure or increase current agricultural yields while reducing the input of fertilisers and pesticides. Two envisioned terpenoid application areas are: (1) the discovery of terpenoid-based interactions involved in increasing crop resilience against abiotic stresses (drought, salinity, nutrient limitation) and biotic stresses (pest and pathogen attacks), and (2) the discovery of new bioactive terpenoid compounds with antimicrobial activity, which can be used to control plant pathogens. The demand for new approaches and compounds is high both in agriculture (EU-ban of many chemical pesticides) and in healthcare (antibiotic resistance, side-effects). However, plants often produce only minute amounts of these valuable chemicals. Thus, the above-mentioned microbial production could provide a solution to these limitations *via* more straightforward, cheaper and more sustainable production of economically, agriculturally and medicinally important terpenoids.

6. Knowledge gaps and questions for future studies

In recent years, technical advances in-omics, as well as advances in analytical methods paved the way for studies on bioecological roles, *i.e.*, bioactive properties and function in chemical interactions, of terpenoids in their natural environments or close-to-natural laboratory setups. Analysis of terpenoid diffusion in soil systems revealed that volatile terpenoids can diffuse in the

rhizosphere environment in the several decimeter range,[233,263] which indicates a wide signaling impact.[264] Transcriptomic and proteomic analyses enable to study how microbial gene expression is affected in response to terpenoid signals during microbial interactions.[100,230] These studies provide insights into the role of terpenoids as signaling molecules, although many questions on how these signals are sensed and transduced at the cellular level are left unanswered. Addressing these questions in combination with analyses of concentrations, at which terpenoids are produced and exert specific bioecological functions in the natural environment, are required for the clarification of ecological roles of microbial terpenoids.

Knowledge of the precise producer in a given ecological systems is a prerequisite for the better understanding of the role of terpenoids in interspecific communication as well as for their application, *e.g.* for the protection of plants against biotic and abiotic stresses.[265] There are still many unknowns on the real terpenoid producer(s) in many cases of plant–microbe interactions and on how plants and insects can benefit from the terpenoids produced by host-associated microbes. Furthermore, many terpenoids have been reported from marine invertebrates such as sponges or octocorals, where they act as a predator deterrents, antifouling or space-competition agents[10,266] and it remains to be clarified whether they are produced by hosts and/or associated microorganisms. Searching for microbial producers of terpenoids coupled with the elucidation of their ecological impact is one of the grand challenges in the fields of aquatic and terrestrial chemical ecology.

7. Author contributions

M. A., P. G., J. S. D. and D. U. conceived the review idea, all authors wrote the manuscript draft and contributed to review/editing.

8. Conflicts of interest

There are no conflicts to declare.

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