











REVIEW

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Biosynthesis of biologically active terpenoids in the mint family (Lamiaceae)

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The Lamiaceae family, the sixth largest among angiosperms, is renowned for its rich diversity of terpenoids, many of which exhibit remarkable bioactivities, including anti-inflammatory, psychoactive, anti-cancer, and antiviral effects. Notable examples with fully elucidated biosynthetic pathways include menthol from peppermint, forskolin from blue spur flower, and carnosol from rosemary. For other key Lamiaceae terpenes—such as the anti-cancer oridonin, the psychoactive salvinorin A, and bioactive marrubiin and vitexilactone—significant progress has been made. This review explores the bioactivity and biosynthesis of Lamiaceae terpenes, with a focus on mono- and diterpenes, while highlighting future research directions.

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1. Introduction

1.1. The Lamiaceae plant family

The Lamiaceae (mint family) are the sixth largest plant family with 7,000 species divided in ten subfamilies and 230 genera.¹ The largest genus alone, *Salvia*, exceeds 900 species.¹ The cosmopolitan distribution across all climate zones is reflected in the traditional use of these plants across many cultures around the globe. Several Lamiaceae species are grown commercially such as lavender and several mint species. They can be recognized by their characteristic flowers having petals fused into an upper lip and a lower lip. Most Lamiaceae species contain essential oils and are aromatic in several parts of the plant. They include culinary herbs like sage, rosemary and thyme, as well as traditional medicines such as motherwort and skullcap. Many of these characteristics are linked to the amazing variety of terpenoids with dozens of backbones. Among these, we can find a wide variety of bioactivities such as psychoactive, anti-cancer, and antiviral activities. Recent advances have greatly improved our understanding of the biosynthesis of monoterpenoids and diterpenoids in various Lamiaceae plants.

1.2. Enzymes involved in Lamiaceae terpene metabolism

Key players in the biosynthesis of terpenes are the terpene synthases, that shape the backbone structure and cytochrome p450 enzymes that decorate the backbone with oxidations.

1.2.1. Terpene synthases. The enzyme family of terpene synthases that are responsible for the first step in terpene biosynthesis have been reviewed before in great detail.^{2,3} The substrate of monoterpene synthases is either geranyl pyrophosphate (GPP) or its isomer neryl pyrophosphate (NPP), the latter is not part of the pathways reviewed here. Most Lamiaceae diterpenes backbones are synthesized, starting from the common precursor geranylgeranyl pyrophosphate (GGPP) **8**, through a combination of a class II diTPS and a class I diTPS.

Class I diTPS initiate cyclization *via* diphosphate abstraction; class II diTPS *via* protonation.⁴ The biosynthesis of diterpenes starts with GGPP **8** (Fig. 1B), that is converted to the specific diterpene backbones either by a bifunctional diterpene synthase (diTPS) or by consecutive activity of diTPS II and diTPS I. All diterpene synthases discussed in this review are monofunctional class I or class II diTPS, the latter are indicated by an asterisk (*) after the enzyme name.

1.2.2. Cytochrome P450s. Cytochrome P450 enzymes (P450s or CYPs) can be classified in families and subfamilies based on their amino acid sequence. In many cases CYPs in the same subfamily carry out similar reactions or use similar substrates. The most relevant P450 families in Lamiaceae terpenoid metabolism are CYP71 and CYP76 (especially subfamilies CYP76AH/AK) (SI Table 1). Besides terpene synthases and CYPs a range of other enzyme types such as acyl transferases play an important role.

1.3. Identification and characterization of enzymes

1.3.1. Genome and transcriptome sequencing. As of July 2025, 84 genome sequences of Lamiaceae plant species were reported – within the genus *Salvia* alone the genome sequences of eight species are available (<https://www.ncbi.nlm.nih.gov/search/all/?term=Lamiaceae>). The accelerated speed of genome sequencing has in recent years facilitated the elucidation of multiple Lamiaceae terpenoid metabolic pathways, while also giving insight into syntenic relationships and gene clusters. Some examples discussed in this article include *Isodon rubescens*,⁵ *Callicarpa americana*,⁶ and *Scutellaria barbata*.⁷ Also, the parallel leaf transcriptome sequencing of 48 Lamiaceae plant species by the Mint Evolutionary Genomics Consortium played an important role in the elucidation of Lamiaceae terpenoid pathways.⁸

1.3.2. Analysis of synteny and gene clusters. The availability of multiple Lamiaceae genomes allows a deeper understanding of syntenic relationships. For instance, Schlecht *et al.* identified a syntenic block containing orthologs of CYP76BK1 across all major lineages of the Lamiaceae that catalyse furan ring formation in clerodane diterpenoids.⁹ Sun *et al.* identified an impressive cluster of tandem-duplicated CYP706V genes that helped to identify the first steps of the oridonin biosynthesis in *Isodon rubescens*. Moreover, they show syntenic relationships of this gene cluster to other species inside and outside of the Lamiaceae family.⁵ Bryson *et al.* showed the syntenic relationship of a miltiradiene biosynthetic gene cluster across the Lamiaceae (see also Chapter 3.1.4).^{10,11}

1.3.3. Enzyme characterization. Once candidate sequences are identified from genome and transcriptome data the classical approach to the elucidation of metabolic pathways from candidate sequences is the stepwise reconstruction in a heterologous host such as yeast and *Nicotiana benthamiana*, as well as microsome assays.^{12,13} This is followed by analysis of GC-MS and LC-MS data of the generated enzyme products considering possible rearrangement reactions during the extraction and analysis.^{13,14}



Dae-Kyun Ro

Dae-Kyun Ro earned his PhD in phenylpropanoid metabolism at the University of British Columbia (UBC), Canada. He then pursued two postdoctoral research projects: one at UBC, focusing on isoprenoid metabolism in pine resin, and another at the University of California, Berkeley, where he worked on synthetic biology for artemisinin production in yeast. Currently, he is a professor at the University of Calgary, Canada. His research

centers on the biochemistry of sesquiterpene lactones, medicinal diterpenes, and natural rubber. In recent years, he has expanded his focus to include the development of novel agricultural traits in legumes.



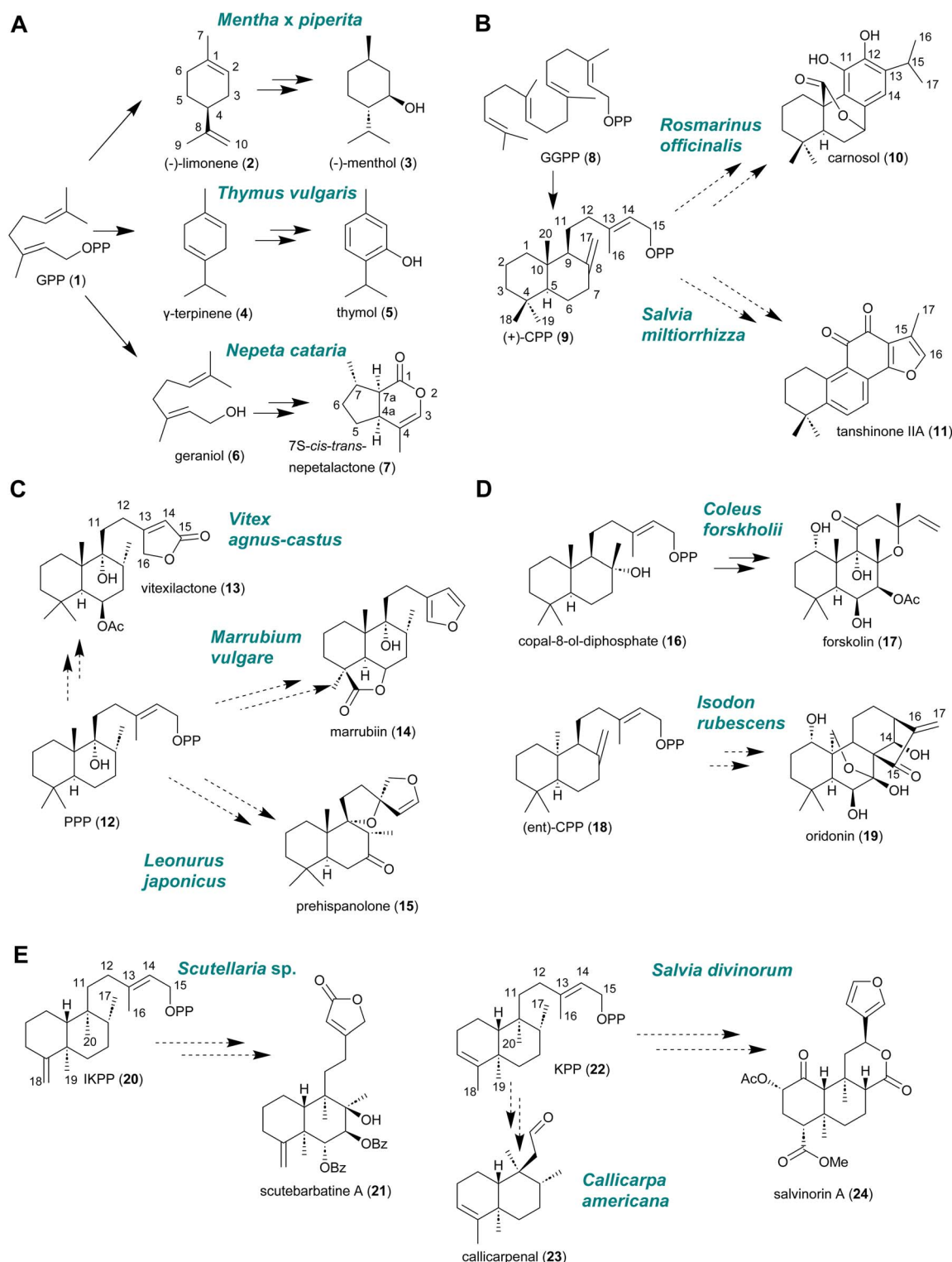


Fig. 1 Overview of Lamiaceae terpenoid pathways. (A) Biosynthesis monoterpenes menthol **3**, thymol **5**, and (7S) *cis-trans* nepetalactone **7**. (A–E) Biosynthesis of diterpenes. (B) Biosynthesis of carnosol **10**, and tanshinone IIA **11**. (C) Biosynthesis of vitexilactone **13**, marrubiin **14** and prehispanolone **15**. (D) Biosynthesis of forskolin **17** and oridonin **19**. (E) Clerodane biosynthesis of scutebarbatine A **21**, callicarpenal **23**, and salvinorin A **24**. GPP: geranyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; CPP: copalyl pyrophosphate; PPP: peregrinyl pyrophosphate; IKPP: isokolavenyl pyrophosphate; KPP: kolavenyl pyrophosphate.



1.4. Metabolic compartmentalization

The mono- and diterpenes discussed in this review are typically derived from IPP and DMAPP units from the plastid-localized non-mevalonate pathway.^{15,16} There has, notably, been evidence for a metabolic cross-talk between mevalonate and non-mevalonate pathway.¹⁷ In most cases the terpenes we present here are produced and stored in glandular trichomes, such as in rosemary, thyme, cat mint and monk's pepper.^{5,18–20} These trichomes have secretory cells that are veritable terpene production machineries that transfer the terpenes in the subcuticular space where they can be stored without impairing the functions of the secretory cells themselves.²¹ However, also other cells and tissues can be involved, such as in blue spur flower, where forskolin is produced in cork cells in the roots.²²

1.5. Structure of the review

In this comprehensive review we cover the research of the last two decades. There are no detailed reports on the biosynthesis of Lamiaceae terpenes yet, however there is an excellent review on the biosynthesis of labdane-related diterpenes that covers some of the aspects discussed here.²³ In Chapter 2, we describe the biosynthesis of these Lamiaceae monoterpenes from the common precursor geranyl-pyrophosphate (GPP) **1** (Fig. 1A). Key progress includes unravelling the pathways for monoterpenoids in peppermint (*Mentha x piperita*) and spearmint (*Mentha spicata*),²⁴ oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*),¹⁸ as well as nepetalactones in catnip (*Nepeta cataria*),²⁵ and cat thyme (*Teucrium marum*).²⁶

One of the earliest examples of the complete elucidation of a complex metabolic pathway leads from GPP **1** via the terpene precursor (–)-limonene **2** to menthol **3**.²⁴ More recently, a metabolic pathway via γ -terpinene **4** to thymol **5** was discovered.¹⁸ Another intriguing group of monoterpenes found throughout the Lamiaceae are the iridoids, which show a fascinating variety of stereochemistries. Today, we know the biosynthesis of iridoids of several conformations that share the common precursor geraniol **6**, such as 7*S*-cis-trans-nepetalactone **7**.

In Chapter 3, we describe the biosynthesis of bioactive Lamiaceae diterpenes. First, we summarize the diterpenes derived from labdane-related backbones (Fig. 1B–D). The biosynthesis of the (+)-CPP **9** (Fig. 1B) derived antioxidative compounds carnosol **10** in rosemary and several tanshinones, such as tanshinone IIA **11** in sage was completely elucidated in recent years. For several Lamiaceae diterpenes derived from peregrinyl pyrophosphate (PPP) **12** (Fig. 1C) the first biosynthetic steps have been found in several plants. These include vitexilactone **13** in monk's pepper (*Vitex agnus-castus*),²⁰ marrubiin **14** in horehound (*Marrubium vulgare*),²⁷ and prehispanolone **15** in Chinese motherwort (*Leonurus japonicus*).²⁸ In blue spur flower (*Coleus forskohlii*) the biosynthesis of the copal-8-ol-pyrophosphate **16** derived cAMP booster forskolin **17** (ref. 29) (Fig. 1D) is completely elucidated. This pathway may provide some valuable insight into the biosynthesis of (ent)-CPP **18** derived anticancer compound oridonin **19** in the closely related plant dong-ling-cai (*Isodon rubescens*),⁵ where initial steps were recently found. In the second part of Chapter 3 we elaborate the biosynthesis of clerodane-type diterpenes (Fig. 1E).

These include the isokolavenyl pyrophosphate (IKPP) **20** derived scutebarbatine A **21** in skullcap (*Scutellaria* sp.) as well as kolavenyl pyrophosphate (KPP) **22** derived callicarpenal **23** in American beautyberry (*Callicarpa americana*),⁶ and the psychoactive salvinin A **24** in divine sage (*Salvia divinorum*).³⁰

2. Monoterpenes

2.1. (Aromatic) monoterpenes

Mint and thyme are herbal plants that have been used since ancient times for their pleasant flavors as food ingredients or as a remedy for various diseases. Within the Nepetoideae, both genera *Mentha* and *Thymus* belong to the tribe *Mentheae*, subtribe *Menthinae*, indicating their close relationship.^{8,31} They produce essential oils (EO) rich in monoterpenes and monoterpene derivatives. The EO is stored in peltate glandular trichomes³² and its constituents are mainly responsible for the biological activities of the respective plant extracts.³³

2.1.1. Monoterpenes in the *Mentha* genus. *Mentha x piperita* (peppermint) and *Mentha spicata* (spearmint) produce the economically important monoterpenes menthol **3** and (–)-carvone **31**, respectively. Due to their fresh and spicy scents, menthol and (–)-carvone are ingredients in oral care products or chewing gums and peppermint oil gained importance as a remedy for irritable bowel syndrome because of its spasmolytic activities.^{34,35} (–)-Menthol is furthermore used as pain reliever due to its ability to activate an ion channel in cold-sensitive neurons (TRPM8). Therefore, it is used in external applications as counter-irritant in cases of e.g. neuropathic pain or headaches.^{36,37}

These two *p*-menthane-type monoterpenes are produced in stereospecific multiple step pathways, both originating from the (–)-limonene **2** enantiomer, which is produced in both peppermint and spearmint from GPP **1** by (–)-limonene synthases (Fig. 2A).^{38,39} Subsequent oxygenation is catalyzed by regiospecific limonene hydroxylases (LS), which hydroxylate (–)-limonene either at C-3 (peppermint) or C-6 (spearmint). These reactions define the oxygen position throughout the whole pathway to (–)-menthol and (–)-carvone, respectively. In general, the enzymes from the CYP71D subfamily play an important role in the first oxidation step following the cyclization to the monoterpene backbone.

2.1.2. (–)-Menthol biosynthesis in peppermint. Menthol was first isolated in the 18th century by Hieronymus Gaubius⁴⁰ and many chemists have worked on unravelling its structure,⁴¹ until the recent structural determination by Egawa *et al.*⁴² Various strategies for the chemical synthesis of menthol have been published; a good overview is provided in a recent review paper that also describes menthol production processes of the companies Symrise and BASF.⁴³ In peppermint, (–)-limonene is hydroxylated by P450 enzymes (CYP71D13 or CYP71D15) at C-3 to yield (–)-trans-isopiperitenol **25**.⁴⁴ Subsequently, (–)-trans-isopiperitenol dehydrogenase (ISPD) forms (–)-isopiperitenone **26**,⁴⁵ which is metabolized by isopiperitenone reductase (iPR) into (+)-cis-isopulegone **27**.⁴⁶ (+)-Pulegone **28** is then formed by isopulegone isomerase (iPI)⁴⁷ and further metabolized to (–)-menthone **29** by (+)-pulegone reductase (PR).⁴⁶ Finally,



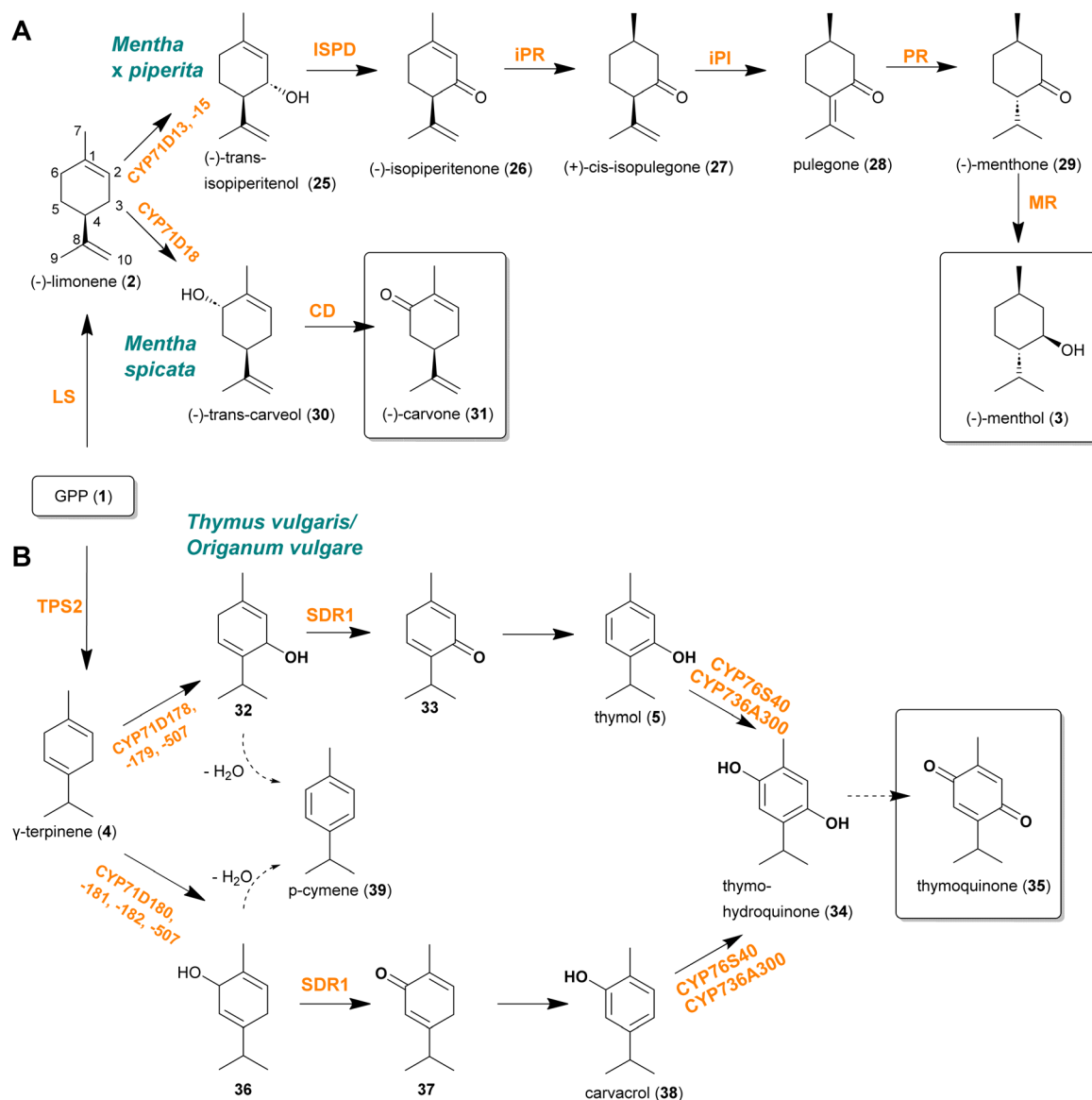


Fig. 2 Biosynthetic pathway leading to monocyclic monoterpenes in Lamiaceae species (A) biosynthetic pathway leading to menthol **3** in peppermint (*Mentha x piperita*) and carvone **31** in spearmint (*Mentha spicata*). (B) Biosynthetic pathway leading to thymol **5**, carvacrol **38** and thymoquinone **35** in thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*). LS: limonene synthase; ISPD: isopiperitenol dehydrogenase; iPR: isopiperitenone reductase; iPI: isopulegone isomerase; PR: pulegone reductase; CD: carveol dehydrogenase; SDR1: short-chain dehydrogenase.

(-)-menthol **3** is formed by menthone reductase (MR)⁴⁸ (Fig. 2A). All enzymes and their respective catalytic activities have been summarized in Croteau *et al.*, 2005.⁴⁹ Immunocytochemical localization studies showed that the pathway is organized in different cell compartments of the peltate glandular trichome secretory cells. According to these studies, the intermediates are transported from leucoplasts to the ER-membrane, further to the mitochondria and cytosol in a very efficient way. For example, the intermediates (-)-trans-isopiperitenol and (-)-isopiperitenone are found only in trace amounts in the EO, so the authors speculate that hydroxylation of limonene by the limonene-3-hydroxylases increases water solubility of the lipophilic compound at the ER and might enable efficient diffusion of isopiperitenol into mitochondria,

next to the (-)-trans-isopiperitenol dehydrogenase generating a concentration gradient.^{49,50}

2.1.3. (-)-Carvone biosynthesis in spearmint. In spearmint, (-)-limonene **2** is hydroxylated at C-6 by CYP71D18 to yield (-)-trans-carveol **30** (ref. 44) which is oxidized to (-)-carvone **31** by (-)-trans-carveol dehydrogenase (CD)⁴⁵ (Fig. 2A). A study from Schalk and Croteau⁵¹ investigated the regiospecificity of CYP71D13 from peppermint and CYP71D18 from spearmint in order to find the enzyme structures that determine the hydroxylation site of (-)-limonene. Due to the high amino acid sequence identity of 70%, they could show that there is one crucial amino acid position in the active center of the P450 enzymes, located 5 amino acids downstream the ExxR sequence motif by domain swapping experiments and site-



directed mutagenesis. The ExxR motif is situated in helix K of the P450 enzyme and as part of the E-R-R triad it is thought to facilitate heme binding and overall stabilization of the meander region of the enzymes.^{52,53} When altering the phenylalanine at position 363 in CYP71D18 into an isoleucine, which is present in CYP71D13 at the corresponding position, the hydroxylation site of (–)-limonene by the mutant (F363I) was altered completely from C-6 to C-3. However, the respective opposite mutation in CYP71D13 (I364F) resulted in an inactive enzyme due to compromised substrate binding orientation and lower binding affinity.

2.1.4. Medical applications of thyme essential oil. The essential oil (EO) of *Thymus* species is the most important commercial resource for the monoterpenes thymol and carvacrol.⁵⁴ Because of the antibacterial and spasmolytic activities of these monoterpenes, thyme extracts are used in many pharmaceuticals, especially in medications for the respiratory system.⁵⁵ Also, they were shown to increase ciliary activity.⁵⁶ Thymohydroquinone and thymoquinone are related oxidation products that are present in thyme EO in small amounts.⁵⁷ Both of these monoterpene derivatives show a wide range of possible applications due to their anti-inflammatory, antioxidant or anti-cancer activities.^{58,59}

2.1.5. Thymol and carvacrol biosynthesis in oregano and thyme. Thymol was originally isolated by Caspar Neumann in 1719.⁶⁰ The formula,⁶¹ and structure⁶² were elucidated in the late 19th century. The biosynthesis of the aromatic backbones of thymol and carvacrol and the subsequent oxidation to thymohydroquinone **34** (Fig. 2B) was elucidated in a recent study.⁵⁷ The initial steps are realized by a similar sequence of enzymes and catalyzed reactions to those that have been reported for (–)-menthol and (–)-carvone biosynthesis in *Mentha* species. The activity of a monoterpene synthase is followed by a hydroxylation of a P450 enzyme from the CYP71D subfamily and an oxidation by a short-chain dehydrogenase resulting in an allylic ketone. In detail, γ -terpinene **4** is formed from GPP **1** by a monoterpene synthase (TvTPS2) and then hydroxylated by P450 enzymes at either C-3 (CYPD178, -179 or -507) or C-6 (CYP71D180, -181, -182, or -507) to form a dienol intermediate **32** & **36**. This intermediate is captured by a short-chain dehydrogenase (SDR1) to form the respective allylic ketone intermediate **33** & **37**, which rearranges to thymol **5** and carvacrol **38**, respectively, by a keto–enol tautomerism. Two P450 enzymes (CYP76S40 and CYP736A300) were identified to perform a second hydroxylation to yield thymohydroquinone, **34**, which oxidises to thymoquinone **35** either spontaneously or by enzymatic catalysis (Fig. 2B). However, an enzyme responsible for the conversion of thymohydroquinone to thymoquinone has not been identified in this study. It is likely that this reaction happens non-enzymatically during storage of the EO in the peltate glandular trichomes. Despite the original hypothesis, *p*-cymene **39** is not an intermediate of the pathway and does not result from oxidation of γ -terpinene. The common co-occurrence of this aromatic monoterpene in EO containing thymol or carvacrol more likely results from an elimination reaction of the dienol intermediates **32** & **36** of the pathway leading to the phenolic monoterpenes. These intermediates are

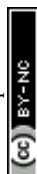
unstable in aqueous conditions and can easily rearrange to *p*-cymene by releasing a water molecule (Fig. 2B). It should be noted that *p*-cymene can also be formed from γ -terpinene by autooxidation in the presence of air (oxygen). It was hypothesized that the amount of *p*-cymene being released as side-product is dependent on how the P450 enzymes and SDRs catalyzing the hydroxylation of γ -terpinene and subsequent oxidation of the dienol intermediate interact within the plant cells. If the proteins are well associated, it is less likely for the intermediate to be released into the aqueous surrounding than in cases in which the enzymes are located more distantly.⁵⁷ They hydroxylate γ -terpinene either at C-3 (CYPD178, -179 or -507) or C-6 (CYP71D180, -181, -182, or -507). Despite sequence similarities of minimum 80% between groups, the amino acids responsible for the stereospecificity have not been identified so far. In 2022, the first genome of a thyme species, *Thymus quinquecostatus*, was reported and enabled the confirmation of several biosynthetic clusters containing TPS-, CYP-, and SDR enzymes on genomic level.⁶³ This result emphasizes that the interaction of these enzyme classes is crucial in terpenoid biosynthesis in thyme species.

2.2. Lamiaceae iridoids

2.2.1. Occurrence and medical applications of Lamiaceae iridoids. Iridoids are non-canonical monoterpenoids that are mainly found in the Asterid group of flowering plants. They have insect repellent properties and are considered important plant defence compounds. In addition, volatile iridoids such as nepetalactone and dolichodial **54** are attractive to wild and domestic cats and induce euphoric behaviour. There are several stereoisomers of nepetalactone, such as (7*S*)-*cis-trans*-nepetalactone **7**. Nepetalactone and related iridoids, which were first isolated from catnip oil in 1941,⁶⁴ are characteristic constituents of *Nepeta cataria* (catnip), *N. mussinii* (catmint) and *Teucrium marum* (cat thyme), which all belong to the mint family (Lamiaceae).^{25,26}

Iridoid biosynthesis has been intensively studied in recent years and starts with the formation of geraniol **6** from GPP **1**, followed by a series of oxidations, reductions and other modifications. In this chapter, we summarize recent findings on the biosynthesis of nepetalactone and related iridoid volatiles in *Nepeta* and *Teucrium*.

The iridoids, found throughout the Lamiaceae, are “irregular” monoterpenes with a characteristic fused ring structure (Fig. 3). Iridoid biosynthesis was lost in the large Nepetoideae sub-family of the Lamiaceae,⁸ but careful analysis suggests that iridoids re-evolved in the *Nepeta* genus.⁶⁵ Nepetalactones are the active ingredients that cause cats to strongly react to *Nepeta* species, plants that are aptly nicknamed catnip or catmint.⁶⁶ Additionally, nepetalactones appear to deter insect pests, prompting application of extracted *Nepeta* oils as a natural and safe insect repellent.⁶⁷ It is not clear how the stereochemistry of nepetalactone isomers impacts the observed biological activities, though some studies suggest that mixtures of stereoisomers are required for optimal effects.⁶⁸ The biosynthesis of nepetalactones in *Nepeta* has been well studied (Fig. 3). The



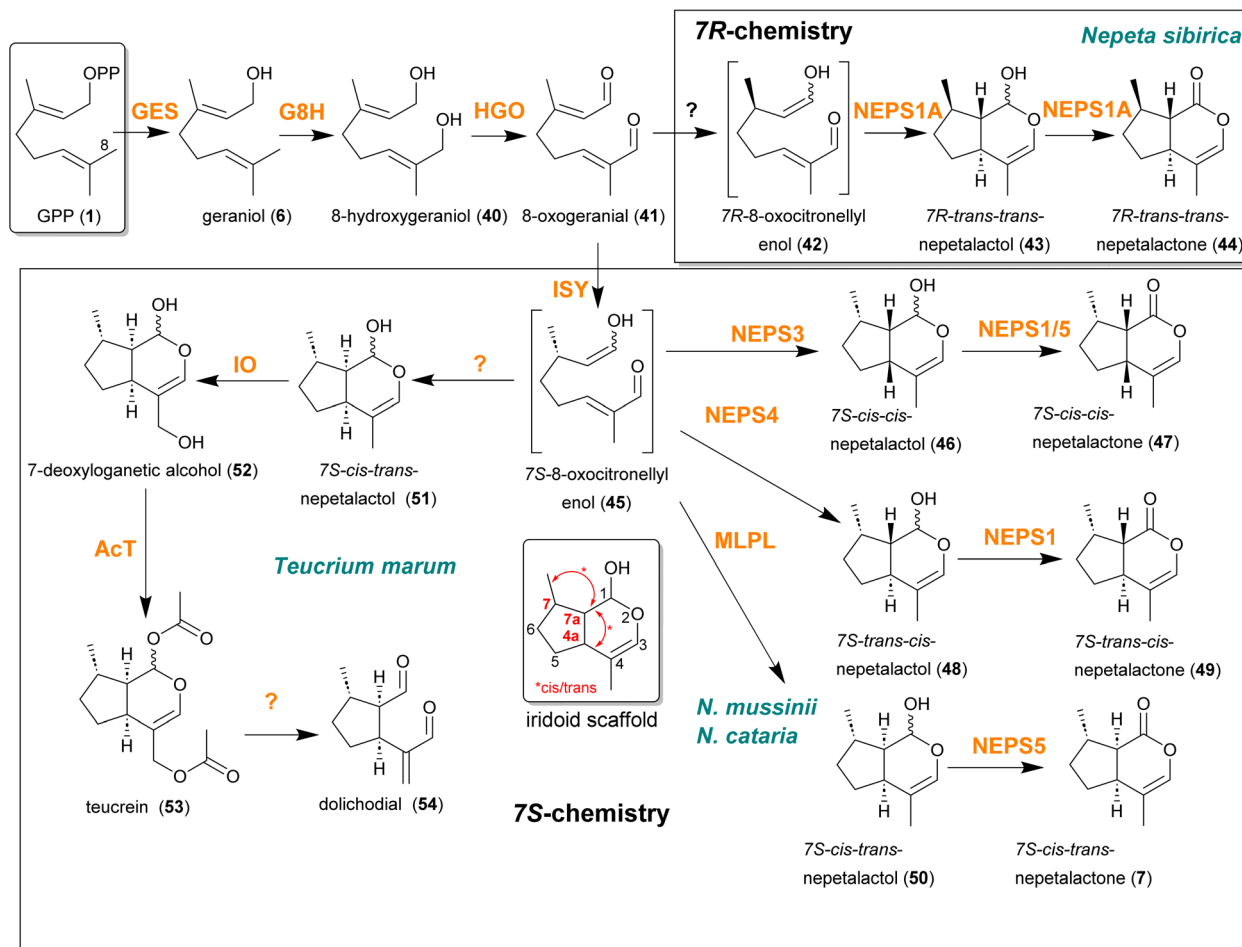


Fig. 3 The biosynthesis of volatile iridoids in *Nepeta* spp. and *Teucrium marum*. GES, geraniol synthase (terpene synthase); G8H, geraniol 8-hydroxylase (cytochrome P450); HGO, 8-hydroxygeraniol oxidoreductase (medium chain alcohol dehydrogenase); ISY, iridoid synthase (short chain alcohol dehydrogenase); NEPS, nepetalactol-related short chain alcohol dehydrogenase; MLPL, major latex protein-like; IO, iridoid oxidase (cytochrome P450); AcT, iridoid acetyltransferase (BAHD acyltransferase). Question marks indicate not yet identified enzymes. While enzymes of the core pathway including GES, G8H, and HGO are orthologous in *Nepeta* and *Teucrium*, ISY activity emerged independently in the two genera. For compounds **43–50** the names from the literature are given, that refer to the stereo-chemical configuration of the C7 methyl group and the 7a–4a bridge. An alternative description of their stereochemistry would be: 7R,4aS,7aS-nepetalactol (7R-trans-trans-nepetalactol) **43**, 7R,4aS,7aS-nepetalactone (7R-trans-trans-nepetalactone) **44**, 7S,4aR,7aS-nepetalactol (7S-cis-cis-nepetalactol) **46**, 7S,4aR,7aS-nepetalactone (7S-cis-cis-nepetalactone) **47**, 7S,4aS,7aS-nepetalactol (7S-trans-cis-nepetalactol) **48**, 7S,4aS,7aS-nepetalactone (7S-trans-cis-nepetalactone) **49**, 7S,4aS,7aR-nepetalactol (7S-cis-trans-nepetalactol) **50**.

storage and biosynthesis of these compounds was shown in *N. racemosa* to be localized to the trichomes on the leaves.⁶⁹ This knowledge allowed subsequent identification of the biosynthetic enzymes and corresponding genes by proteomic analysis of isolated trichomes of *N. cataria* and *N. mussinii*.^{65,70} Like all monoterpenes, iridoids derive from GPP.

2.2.2. Biosynthesis of the common intermediate 8-oxogeraniol. All iridoids discussed in this chapter share the common intermediate 8-oxogeraniol **41**. GPP **1** is subject to the action of geraniol synthase (GES), a standard monoterpene synthase that generates geraniol **6** from GPP through a canonical cationic intermediate. Geraniol is then hydroxylated by a cytochrome P450 (CYP76), geraniol 8-hydroxylase (G8H, also referred to in older literature as G10H). The resulting diol 8-hydroxygeraniol **40** is then oxidized to the corresponding di-

aldehyde 8-oxogeraniol **41** by a zinc and NAD(P)⁺ dependent medium chain alcohol dehydrogenase, hydroxygeraniol oxidase (HGO).

2.2.3. Branching point to iridoids with 7R and 7S stereochemistry. This reactive di-aldehyde **41** is subjected to a stereo-selective 1,4 reduction by a short chain alcohol dehydrogenase, iridoid synthase (ISY). Characterized ISY enzymes from *Nepeta* stereoselectively catalyze this reduction to yield 7S-8-oxocitronellyl enol **45**. Iridoids with 7R stereochemistry have been observed in *N. sibirica*,⁷¹ but no ISY with the corresponding *R* stereoselectivity has been identified in any *Nepeta* species, though a 7R-ISY has been identified in an evolutionarily distant plant (snapdragon).⁷² The ISY product 8-oxocitronellyl enol **42** can cyclize spontaneously, albeit inefficiently, to form the nepetalactol scaffold.⁷⁰ However, analysis of the trichome



localized proteins in *N. cataria* and *N. mussinii* led to the discovery of cyclases that act in partnership with ISY to increase the efficiency and stereoselectivity of the cyclization.^{65,70} These cyclases presumably act by conformationally orienting 8-oxocitronellyl enol.

2.2.4. Biosynthesis of *Nepeta* iridoids. A protein of the major latex protein (MLP) family leads to the formation of 7*S*-*cis*-*trans*-nepetalactol **51**. Cyclases that have homology to short chain alcohol dehydrogenases catalyze cyclization to 7*S*-*cis*-*cis*-nepetalactol **46** (NEPS3) and 7*S*-*trans*-*cis*-nepetalactol **48** (NEPS4). These nepetalactol isomers are oxidized to the corresponding nepetalactones **47** & **49** by oxidoreductases (NEPS1, NEPS5), proteins that have high sequence identity to NEPS3 and NEPS4. The fourth possible 7*S* stereoisomer, 7*S*-*trans*-*trans*-nepetalactol, has not been identified in nature, but *N. sibirica* produces 7*R*-*trans*-*trans*-nepetalactone **44**. A short chain alcohol dehydrogenase (NEPS1A) that catalyzes the stereoselective formation of 7*R*-*trans*-*trans*-nepetalactol **43** from 7*R*-8-oxocitronellyl enol **42** was identified from this species.⁷¹ NEPS1A also catalyzed oxidation of this product to 7*R*-*trans*-*trans*-nepetalactone **44**. In contrast to the volatile nepetalactones produced by *Nepeta*, the vast majority of iridoids produced by the Lamiaceae are glycosylated.⁸

2.2.5. Biosynthesis of *Teucrium* iridoids. However, *Teucrium marum*, a member of the Ajuga sub-family, also produces a volatile terpenoid, namely dolichodial **54**. Recent work has partially elucidated the biosynthesis of this compound.²⁶ An ISY along with an unidentified cyclase leads to the formation of 7*S*-*cis*-*trans*-nepetalactol **51**. A cytochrome P450 (CYP76), iridoid oxidase (IO), hydroxylates this product to **52**, and an acyl-transferase (AcT) adds two acetyl groups leading to teucrein **53** (Fig. 3). Elimination of the acetyl groups is proposed to lead to dolichodial **54**. Notably, it appears that iridoid biosynthesis was lost in the Nepetoideae through loss of the ISY gene, which then re-emerged *via* parallel evolution in the *Nepeta* genus.⁶⁵ Consequently, the ISY from *T. marum* and *Nepeta* appeared to have evolved independently.²⁶ The iridoid pathway in the Lamiaceae provides a fascinating window on the evolution of complex metabolic pathways in plants.

3. Diterpenes

Here, we describe metabolic pathways that proceed *via* two GGPP-derived **8** (Fig. 1B) backbone types: First, the labdane-related backbones of (+)-CPP **9** (Fig. 1B), peregrinyl pyrophosphate (PPP) **12** (Fig. 1C), 8-hydroxy-CPP **16** or *ent*-CPP **18** (Fig. 1D). Second, the (neo-)clerodane backbones of IKPP **20**, and KPP **22** (Fig. 1E). Most of the following synthesis steps involve cytochrome P450s enzymes, especially from the CYP families CYP71, CYP76 and CYP706.

3.1. Carnosol in rosemary (*Rosmarinus officinalis*) and tanshinones in *Salvia miltiorrhiza*

3.1.1. Occurrence of carnosol and tanshinones. The CPP-derived abietane-type diterpenoids (Fig. 1B) are abundant natural products in the Lamiaceae family. Among them, the (+)

CPP **9** derived carnosic acid (CA) **59** is one of the most well-known and is produced in the genera *Salvia* (sage and rosemary), *Lepechinia* (pitcher sage), *Oreganum* (oregano), and *Thymus* (thyme).^{73–75} It may also be present in *Ocimum* (basil) and *Hyssopus* (hyssop) because derivatives have been found in these species which are likely produced from CA.^{73,76} CA was first discovered in extracts of *Salvia officinalis* in 1962.⁷⁷ CA is produced in trichomes which allow it to accumulate up to 4–10% on weight basis of air-dried leaves.^{78–81} Exposed to air, CA oxidizes spontaneously to carnosol (CO, **10**), a 20,7 β -olide derivative, that was detected in leaves of sage species, rosemary, oregano, basil (*Ocimum basilicum*) and thyme (*Thymus vulgaris*).^{73,80,81} Both CA **59** and CO **10** have an *ortho*-diphenolic structure which is responsible for the antioxidant activity of CA.⁸² In addition, both possess diverse biological activities including neuro-protective, antimicrobial and anti-inflammatory properties. For more detail on the diverse activities of CA and CO as well as clinical trials, we refer to previous reviews.^{83–86}

In industry, rosemary extracts are used as food preservatives (food additive E392 in the European Union and CNS 04.017 in China) or in cosmetics, and the activity is ascribed to CA and CO. Tanshinones such as tanshinone I **66** and tanshinone IIA **11** are close relatives to CA and CO but have a furan D-ring. They are exclusively produced in sage species, *e.g.*, in *Salvia miltiorrhiza* (Chinese danshen), and in the *Salvia* subgenus *Perovskia*.⁸⁷ Tanshinones were first isolated from danshen in 1934 by Nakao and Fukusima.⁸⁸ Roots of *S. miltiorrhiza* have been used for centuries in Chinese traditional medicine to treat coronary artery disease and other cardiovascular and cerebrovascular disorders.^{89–91} Additional health-promoting activities make tanshinones attractive for medical applications including anti-cancer,⁹² anti-HIV⁹³ and hepatoprotective properties.⁹⁴ Accordingly, multiple clinical trials are under way; ten have been published in the last five years alone.

3.1.2. The common pathway to 11-hydroxyferruginol. The biosynthesis of the tricyclic diterpene backbone of CA, CO and tanshinones (Fig. 4) starts with the cyclization of GGPP, **8** to CPP **9** by CPP synthase and further by a kaurene synthase-like (KSL) enzyme to miltiradiene **55**.^{79,95} It is likely that miltiradiene spontaneously oxidizes to abietatriene **56**.⁹⁶ *S. miltiorrhiza* has five CPS* and two KSL of which only SmCPS1*, SmCPS2* and SmKSL1 are involved in tanshinone biosynthesis.⁹⁷ The downstream oxidative decoration of the miltiradiene or abietatriene backbone is dominated by cytochrome P450 enzymes of the CYP71 clan. Many of them accept multiple substrates, thus contribute to the structural diversity of abietane diterpenes in rosemary and diverse sage species.^{98,99} A ferruginol synthase (CYP76AH1) in *S. miltiorrhiza* initiates a series of oxidation reactions *via* ferruginol **57**.¹⁰⁰ This catalytic hydroxylation at position C12 can be seen in rosemary and other sage species too, but it is part of a sequential oxidation that leads to 11-hydroxy ferruginol **58** with hydroxyl groups at positions C11 and C12 through the activity of hydroxyferruginol synthase (CYP76AH2-24).^{81,101} In *S. miltiorrhiza*, oxidation of C11 is carried out separately by CYP76AH3.^{98,102,103} All CYPs that oxidize position C11 perform hydroxylation at C7 too.^{81,98,103} The



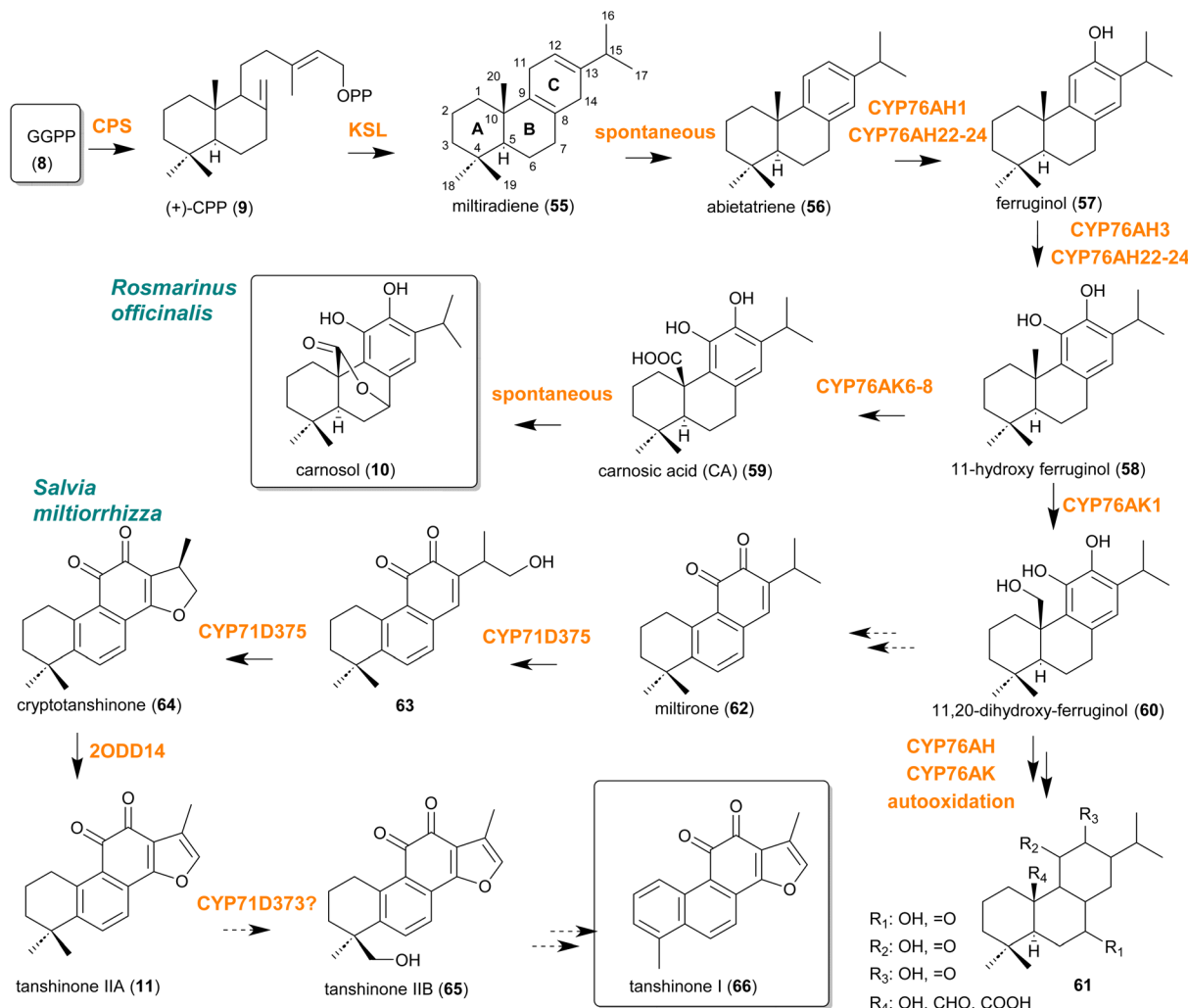


Fig. 4 Biosynthesis of carnosol **10** and carnosic acid **59** in rosemary as well as tanshinones (**11**, **64–66**) in sage. 2ODD14: 2-oxo-glutarate dependent dioxygenase. CPS*: copalyl pyrophosphate synthase. KSL: kaurene synthase like.

ortho-diphenolic ring with hydroxylated C11 and C12 oxidizes spontaneously to the quinone form,⁸¹ whereas the conversion to the keto form of the OH group at C7 is catalyzed enzymatically.¹⁰⁴ C20 is another position which is oxidized in both CA/CO and tanshinone pathways.

3.1.3. Branching of the carnosol and tanshinone pathway. In the tanshinone producing species *S. miltiorrhiza* CYP76AK1 (and CYP71D411) introduce a hydroxyl group that yields 11,20-dihydroxy-ferruginol **60**, whereas in the CA producing species (rosemary and sage) CYP76AK6-8 further oxidizes to the aldehyde and the carboxy group^{81,101,103,104} leading to CA **59**. The latter functionalization is specific to CA-producing species and apparently does not occur in tanshinones. In *S. miltiorrhiza*, two more CYP76AK exist, namely CYP76AK2 and CYP76AK3 which are known to contribute to tanshinone biosynthesis.¹⁰⁵ However, their specific role remains unclear.¹⁰⁵ The promiscuity of the involved CYP76AHs and CYP76AKs as well autooxidation allow diverse combinations of oxidations at C7, C11, C12 and C20 resulting in a complex mix of structurally diverse abietane diterpenes.^{98,106}

3.1.4. Tanshinone biosynthesis in *Salvia miltiorrhiza*. Miltirone **62** is an important intermediate in tanshinone biosynthesis.¹⁰⁶ The total synthesis of this compound has been achieved but the biosynthesis in plants remains elusive.¹⁰⁷ It is not clear, yet, how one of the precursors (most likely 11,20-dihydroxy-ferruginol) is converted to miltirone. It would require demethylation to lose the C20 and aromatization of the B ring; both are believed to happen in a coupled transformation.¹⁰⁶ However, miltirone serves as substrate in a key reaction to create the D-furan ring. CYP71D375 (and CYP71D373) first hydroxylates its substrate at C16 to form **63** and then performs ring closure with C14 *via* a 14,16-ether (hetero)cyclization.¹⁰³ The resulting cryptotanshinone **64** is reduced by the 2-oxo-glutarate dependent dioxygenase Sm2ODD14 to tanshinone IIA **11**. The latter can be converted to tanshinone IIB **65** by hydroxylation of C19. Such an enzyme has recently been proposed (SmCYP71D373) but no NMR was provided to prove the stereochemistry as described for SmCYP81C16 that yields isotanshinones by oxidation at C18.^{108,109}

Downstream, tanshinone I **66** biosynthesis requires the loss of a methyl group at C4 (and aromatization of ring A), and oxidation of C18 or C19 might be an intermediate step in this transformation. The sequence of enzymes putatively involved in tanshinone biosynthesis can be extracted from genome and transcriptome data.^{110–113} However, to date, the complete pathway to tanshinones still remains to be elucidated.

3.1.5. Biosynthetic gene clusters in tanshinone biosynthesis.

Biosynthetic gene clusters (BGC) are a common feature of plant specialized metabolism.^{114,115} The physical proximity of genes coding for enzymes involved in the same pathway provide diverse benefits related to fitness, cluster regulation, inheritance, and neofunctionalization. In CA and tanshinone biosynthesis, upstream genes (CPS*, KLS and CYP67AH) reside nearby. For example, a cluster has been identified in rosemary that contains *RoCPS2**, *RoKSL2*, and *RoCYP76AH22*.^{10,11} In *S. miltiorrhiza*, multiple BGCs have been identified,^{103,116,117} reflecting the complexity of tanshinone biosynthesis. *SmCPS1** clusters with *SmCPS2**, *SmKSL1*, *CYP76AH1*, *CYP76AH3* and uncharacterized *SmCYP76AH28*; *SmCPS1** is located close to *CYP76AH12* and *CYP76AH13*; and *SmCYP71D411* resides nearby *CYP71D375* and *CYP71D373*. As the deciphering of the tanshinone pathway progresses, other or expanded BGCs could be uncovered.

3.1.6. Metabolic engineering of carnosic acid and tanshinone biosynthesis.

The interest in CA and tanshinones has spurred efforts to improve their production in different host organisms. One approach targets the original producing plant species with improvement strategies including selection of rosemary varieties with increased CA production as well as manipulation of *S. miltiorrhiza* hairy root cultures that can yield around 3 mg total tanshinones per g dry weight.^{78,118,119} Microbial factories, in particular fungal production platforms, have been exploited as well, with CA titers reaching to over 75 mg L⁻¹ in both *Candida tropicalis* and *Saccharomyces cerevisiae*.^{120,121} The involvement of multiple enzymes (mostly CYPs) with a certain degree of substrate promiscuity leads to a complex metabolic grid, making it challenging to engineer the production of a specific compound. Therefore, protein engineering is required to shift substrate specificity towards single products,¹²² and to improve enzyme kinetics. In addition, proper flux of precursor metabolites is needed to provide sufficient amounts of substrate for downstream abietane diterpene production.^{123,124} The great potential of abietane diterpenes from the Lamiaceae family make elucidation of biosynthetic pathways, pathway engineering as well as production in platform organisms attractive for pharmaceutical and industrial applications. In addition, the promiscuity of involved enzymes provides excellent material to develop new-to-nature metabolites with new bioactivities.¹²⁵ Metabolic engineering to explore the chemical space of miltiradiene-like backbones could also be further elaborated by incorporating site-directed mutagenesis.¹²⁶

3.2. Vitexilactone in monk's pepper (*Vitex agnus-castus*)

3.2.1. Occurrence and medical applications of monk's pepper and vitexilactone.

Monk's Pepper (*Vitex agnus-castus* L.)

is a medicinal plant native to the Mediterranean region.¹²⁷ Belonging to the Lamiaceae family and the Viticoideae subfamily, *V. agnus-castus* has long been known for its therapeutic properties.^{127,128} Particularly its fruits have been used in traditional medicine to alleviate disorders of the menstrual cycle and is still used today as treatment of the premenstrual syndrome.^{128–130} The plant's medicinal effects are largely attributed to its diterpene compounds, with over 100 distinct compounds identified within the genus.¹³¹ These diterpene compounds consist of clerodane-, abietane-, halimane-, isopimarane-, norlabdane-, and predominantly labdane-type backbones.¹³¹ One such compound, vitexilactone **13**, is a labdane-type diterpenoid that accumulates abundantly in the trichomes of the fruits and leaves of *V. agnus-castus* (Fig. 5).¹³² Vitexilactone was first isolated and characterized by H. Taguchi in 1976.¹³³ The structure of vitexilactone is further characterized by a C9 hydroxy group, a five-membered lactone ring and an additional acetyl group on the C6 hydroxy group (Fig. 5).^{132,134}

3.2.2. Vitexilactone biosynthesis.

The biosynthesis of vitexilactone is still not well understood, but a few enzymes have been identified that are likely involved in its formation. The biosynthesis of vitexilactone presumably starts with a class II diterpene synthase (diTPS). *V. agnus-castus* contains three class II diTPS, designated as VacdiTPS1*, 3 and 5, which belong to the angiosperm TPS-c subfamily.^{2,132} Notably, VacdiTPS1* shares the closest relationship with peregrinyl pyrophosphate synthase from *Marrubium vulgare* (MvCPS1*).¹³⁵ Heterologous overexpression of the candidate genes in *Nicotiana benthamiana* revealed that VacdiTPS1* does indeed function as a peregrinol pyrophosphate synthase (Fig. 5).¹³² The identified VacdiTPS3 possesses activity as a *syn*-copalyl pyrophosphate synthase, whereas VacdiTPS5 functions as a KPP synthase.¹³² In addition, three class I diterpene synthases from the TPS-e/f subfamily were identified in *V. agnus-castus*, designated as VacdiTPS2, 4, and 6.^{2,132} Combinatorial assays with respective class II diTPS revealed that VacdiTPS2, in combination with VacdiTPS1*, produces viteagnusin D and 9,13(*R*)-epoxylabd-14-ene, and in combination with VacdiTPS3 vitexifolin A.¹³² Coupling of VacdiTPS6 with the peregrinyl pyrophosphate synthase VacdiTPS1* led to the formation of labda-13(16),14-dien-9-ol, whereas VacdiTPS4 possesses activity as *ent*-kaurene synthase with *ent*-CPP as substrate.¹³² The formation of peregrinyl pyrophosphate (PPP, **12**), a diterpenoid precursor with a labdane backbone and a hydroxy group at C9, by VacdiTPS1* is considered essential for the subsequent biosynthesis of vitexilactone, which likely involves its further stepwise oxidation catalysed by cytochrome P450 monooxygenases (Fig. 5).¹³² Transcriptome analysis of *V. agnus-castus* trichomes from fruits and leaves identified one cytochrome P450 monooxygenase within the CYP76BK subfamily of the CYP76 family and the CYP71 clan.¹³² Heterologous overexpression of the corresponding candidate, VacCYP76BK1, revealed its enzymatic activity in catalyzing the 16-hydroxylation of peregrinyl **67**, the dephosphorylated product of VacdiTPS1*, converting it to labd-13*Z*-ene-9,15,16-triol **68**.¹³² CYP76BK1-catalyzed 16-hydroxylation is thought to be a key step in the biosynthesis of the lactone moiety of vitexilactone.¹³²



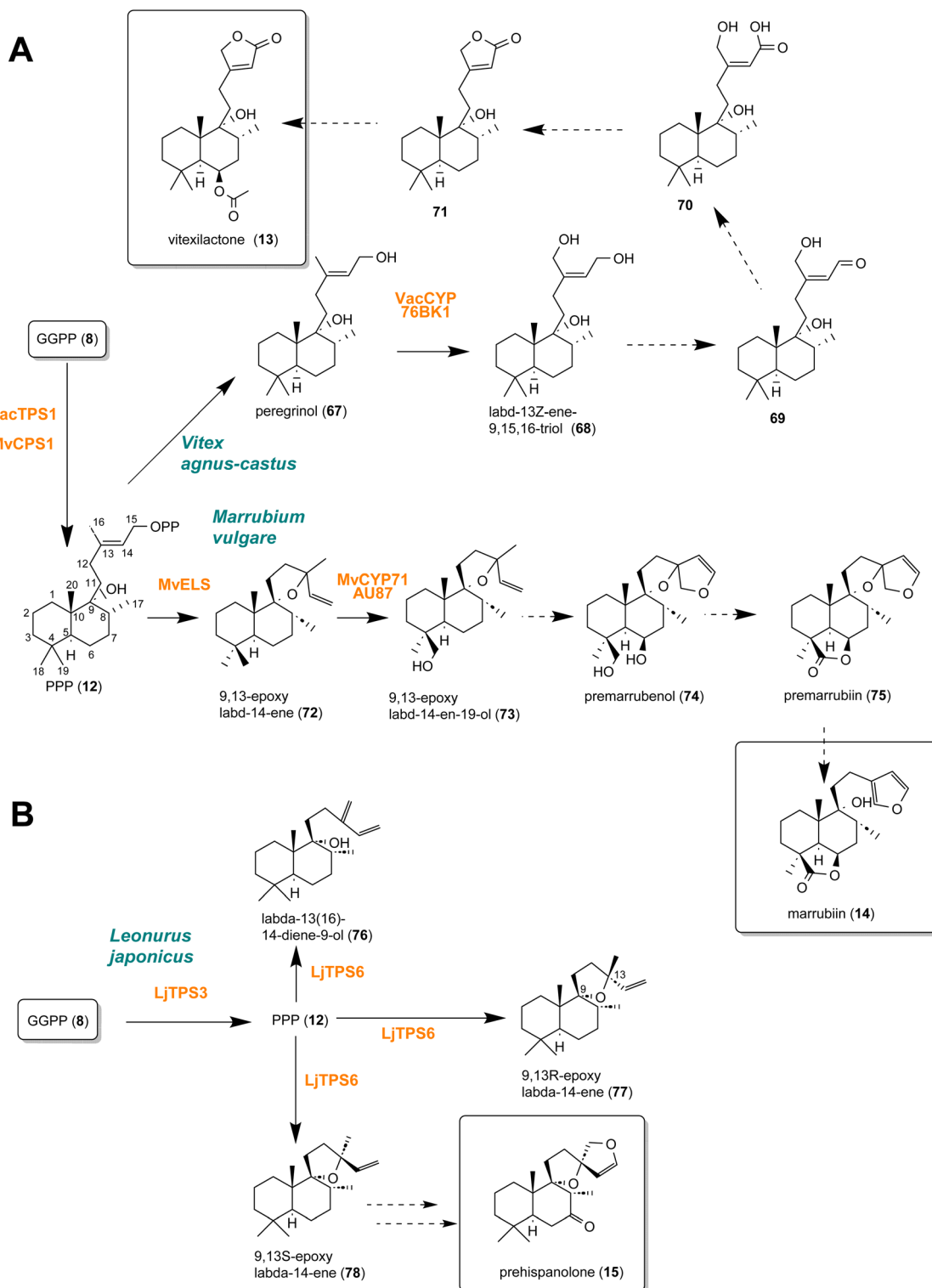


Fig. 5 Biosynthesis of PPP derived diterpenes. (A) Vitexilactone **13** in *Vitex agnus-castus*, marubiin **14** in *Marrubium vulgare* as well as (B) prehispanolone **15** in *Leonurus japonicus*. ELS: 9,13-epoxy-labd-14-ene synthase.

The exact role of peregrinyl as a CYP76BK1 substrate, whether a direct class II diTPS product or an artificial product from the *Nicotiana benthamiana* overexpression system based on endogenous phosphatase activity, remains unclear.¹³² The subsequent steps in the vitexilactone biosynthesis upon formation of labd-

13Z-ene-9,15,16-triol **68**, including further oxidation at position C15 or C16 to intermediates **69** and **70** allowing spontaneous cyclization to the lactone moiety **71**, as well as the addition of the acetyl group, are not yet known and the corresponding enzymes have not been identified (Fig. 5).



3.3. Marrubiin in horehound (*Marrubium vulgare*)

3.3.1. Occurrence and medical applications of white horehound and marubiin. White horehound (*Marrubium vulgare*) is a medicinal plant native to the Mediterranean and Central Asia that has been traditionally used to treat dermatologic and respiratory problems.^{136–138} In addition, *M. vulgare* metabolites and extracts have recently been shown to potentially treat cardiovascular disease, liver disease, and type II diabetes.^{139–141} *M. vulgare* contains a diverse set of specialized metabolites, including aromatic compounds such as flavonoids and other phenylpropanoids, as well as a variety of diterpenoids.¹⁴² One of the pharmacologically active metabolites of *M. vulgare* is the diterpenoid marrubiin **14** that accumulates predominantly in aboveground tissues of the plant such as in leaves and flower calyces.^{135,143–145} The structure of marrubiin was identified by Hollis *et al.* in 1939,¹⁴⁶ and later the stereochemistry was resolved by Appleton *et al.* in 1967.¹⁴⁷ Structurally, the diterpenoid marrubiin is further characterized by a labdane core structure, a furan and γ -lactone moiety, as well as a specific hydroxy group at position C-9 (Fig. 5).^{135,136,138} However, it has been discussed that the characteristic C-9 hydroxy group, which is present in marrubiin and other diterpenoids of the genus, may be formed non-enzymatically during extraction, whereas the native form of the compound is supposed to contain a 9,13-epoxy bridge *in planta*.^{144,148}

3.3.2. Marrubiin biosynthesis. The biosynthesis of marrubiin and other labdane-related diterpenoids in angiosperms is likely mediated by the combinatorial work of class II and class I diTPS, which belong to the TPS-c and TPS-e/f of the angiosperm TPS subfamily.^{2,132,135,149} Transcriptome analysis of *M. vulgare* leaves allowed the identification of three class II diTPS, designated as MvCPS1*, MvCPS2*, and MvCPS3*, and two class I diTPS, designated as MvEKS and MvELS*.^{135,149} Heterologous overexpression of the class II diTPS revealed that MvCPS1* possesses activity as a peregrinyl pyrophosphate synthase, including the regio-specific oxygenation at C-9, which is a characteristic structural feature of marrubiin (Fig. 5).¹³⁵ No enzymatic activity was detected for the identified candidate MvCPS2*, whereas MvCPS3* showed enzymatic activity as (+)-copalyl pyrophosphate synthase.¹³⁵ Combinatorial assays of the identified class I diTPS from *M. vulgare* with corresponding class II diTPS revealed that MvEKS has specific activity as *ent*-kaurene synthase with *ent*-CPP **18** as substrate, whereas the multifunctional MvELS* accepts (+)-CPP, labda-13-en-8-ol pyrophosphate, and peregrinyl pyrophosphate as substrates, catalyzing the formation of miltiradiene, manoyl oxide, as well as labda-13(16),14-dien-9-ol, and 9,13-epoxy-labd-14-ene **72** respectively.^{135,143} The hypothesized pathway and the potential role of the class II and class I diTPS as the first steps in marrubiin formation is supported by the formation of 9,13-epoxy-labd-14-ene **72** as the combined enzymatic product of MvCPS1* and MvELS*, its structural characteristic 9,13-epoxy bridge and by its described occurrence in extracts of *M. vulgare* flowers.^{135,143} Moreover, further transcriptome analysis of *M. vulgare* leaves led in combination with phylogenetic analysis to the identification of a cytochrome P450 monooxygenase of the

CYP71 family, designated as MvCYP71AU87.¹⁴³ Heterologous overexpression of MvCYP71AU87 revealed that the P450 specifically catalyzes the further oxygenation of the MvCPS1* and MvELS* product 9,13-epoxy-labd-14-ene at the positions C-18 and C-19 to 9,13-epoxy-labd-14-en-18-ol and 9,13-epoxy-labd-14-en-19-ol, respectively, as likely intermediates in marrubiin biosynthesis.¹⁴³ Interestingly both enzymatic products of MvCYP71AU87 consists of a 9,13-epoxy group and not a free C-9 hydroxy group.¹⁴³ The hydroxylated products 9,13-labd-14-en-18-ol and 9,13-labd-14-en-19-ol **73** could both not be shown to accumulate as biosynthetic intermediates in *M. vulgare* leaves or flowers, which questions the role of the compounds as natural occurring biosynthetic intermediates in marrubiin biosynthesis.¹⁴³ In addition, further hydroxylation and carboxylation at C-6 and C-19 would be required for the formation of the γ -lactone moiety *via* the intermediate premarrubenol **74**. Further hydroxylation at position C15 and/or C16 would allow for the formation of the furan moiety of premarrubiin **75** and finally marubiin **14** (Fig. 5).¹⁴³ However, corresponding enzymes have not been identified so far.

3.4. Prehispanolone in *Leonurus japonicus*

3.4.1. Occurrence and medical applications of prehispanolone. In the traditional Chinese medicinal herb *Leonurus japonicus* the unusual backbone type of spiro-9,13-epoxy-labdane diterpenoids can be found. One notable example of this bioactive compound class is prehispanolone **15**, first isolated by Hon *et al.* from *Leonurus heterophyllus* extracts in 1991,¹⁵⁰ that possesses anti-platelet aggregation activity.¹⁵¹

3.4.2. Spirolabdane biosynthesis. Wang *et al.*²⁸ describe the diTPS, LjTPS3* and LjTPS6, that can together produce the 13R- and 13S-stereoisomers of 9,13-epoxy-labda-14-ene (**77**, **78**) from GGPP. The downstream pathway to the 13S-bis-spirolabdane backbone **78** (Fig. 5B) of prehispanolone **15** is still unknown. In a first step, the class II diTPS LjTPS3* converts GGPP to PPP **12**. Wang *et al.*²⁸ have also characterized the class II diTPS LjTPS1 producing *ent*-CPP and LjTPS4 producing (+)-CPP. The class II diTPS LjTPS6 was found to accept several substrates and convert them in a complex mix of products. Interestingly, LjTPS6 converted PPP mainly to labda-13(16),14-dien-9 α -ol **76**, and minor amounts of epimers 13S-9,13-epoxy-labda-14-ene and 13R-9,13-epoxy-labda-14-ene *en route* to spirolabdanes, as well as to viteagnusin D. Wang *et al.*²⁸ used homology modeling of LjTPS6 to similar diterpene synthases and docking of PPP to identify candidate sites for mutagenesis. By a tailored modification of amino acids in the catalytic centre the authors were able to shift the product spectrum of LjTPS6:1420G, that produced selectively 13S-9,13-epoxy-labda-14-ene **78**, paving the way for the elucidation of the downstream pathway to and the biotechnological production of prehispanolone **15** in the future.

3.5. Forskolin in blue spur flower (*Plectranthus barbatus*)

3.5.1. Occurrence and medical applications of forskolin. Forskolin **17** (Fig. 6A) is a naturally occurring labdane diterpene renowned for its pharmacological properties. It was first isolated in a screening program by researchers from the Hoechst



AG in 1977 that isolated blood pressure lowering diterpenes from *Coleus* plants.¹⁵² Forskolol is biosynthesized in the roots of the blue spur flower (*Coleus barbatulus* (Andrews) Benth. ex G.Don) and accumulates in the vascular tissue and cork cells of its tuberous roots, reaching concentrations up to $6 \mu\text{g mg}^{-1}$ (yield = 0.01 to 1%, dry weight).¹⁵³ The accumulation of forskolin is not only organ but also species-specific. Despite belonging to a basal lineage within the Lamiaceae family (tribe Ocimeae, subfamily Nepetoideae (Dumort.) Luerss.),¹⁵⁴ *C. barbatulus* appears to be the primary producer of forskolin. However, the precise distribution of this compound within the genus *Coleus* remains unclear.¹⁵⁵ *C. barbatulus* has been used since

ancient times in traditional Hindu and Ayurvedic medicine, primarily for the treatment of heart diseases, respiratory disorders, abdominal pain, and tumors.¹⁵⁶ The medicinal properties of forskolin are attributed to its ability to activate the enzyme adenylyl-cyclase.^{156,157} cAMP acts as a critical intracellular messenger, regulating diverse cellular processes such as metabolism, gene transcription, ion channel activity, and cell growth and differentiation. Recent studies have further highlighted forskolin's role as a potent cAMP booster and its therapeutic potential in treating conditions such as cardiovascular failure,¹⁵⁸ asthma,¹⁵⁹ cancer, HIV,¹⁶⁰ obesity,¹⁶¹ and glaucoma.¹⁶² Forskolol and its water-soluble derivative NKH477 have been

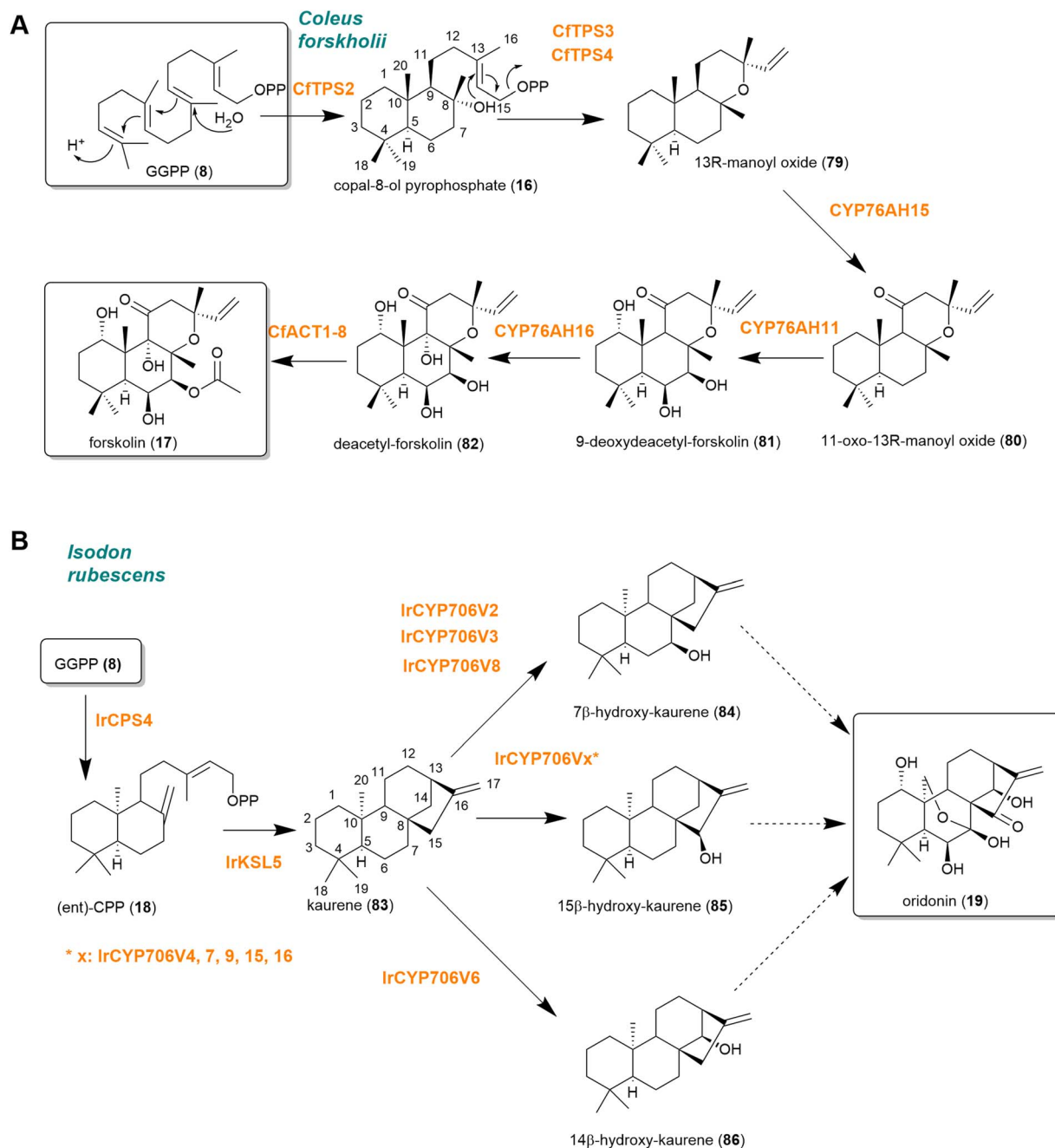


Fig. 6 Biosynthesis of (A) forskolin 17 in *Coleus forskholii* and (B) oridonin 19 in *Isodon rubescens*. ACT: acyl transferase. CPS*: copalyl pyrophosphate synthase.



clinically investigated, demonstrating promising results in the treatment of glaucoma¹⁶² and heart failure.^{163,164}

3.5.2. Forskolin biosynthesis. The biosynthetic pathway of forskolin has been extensively studied over the past decade, culminating in the heterologous production of this valuable plant-derived diterpenoid in engineered *Saccharomyces cerevisiae*.^{22,29,165} Early studies on forskolin biosynthesis identified a set of *CfTPS* candidate genes encoding diterpene synthases (*CfTPS1*, *CfTPS2**, *CfTPS3*, *CfTPS4*, *CfTPS14*, and *CfTPS15*) through transcriptome analysis of *C. barbatius* roots.^{22,149} Functional characterization of five of these genes by Pateraki *et al.*,²² using both *in vitro* and *in planta* assays, revealed that two sequential enzymatic reactions are required for the stereospecific biosynthesis of 13*R*-manoyl oxide **79**, the key precursor of forskolin. These reactions are catalysed by a class II diterpene synthase (*CfTPS2**) and a class I diterpene synthase (*CfTPS3*), converting GGPP **8** into 13*R*-manoyl oxide **79** via the intermediate copal-8-yl-pyrophosphate **16** (Fig. 6A). Notably, this biosynthetic process is localized exclusively in the oil bodies within the root cork cells.²² The conversion of 13*R*-manoyl oxide to forskolin involves six regio- and stereospecific monooxygenations (cytochrome P450s) and a single regiospecific acetylation.¹⁶⁶ These downstream biosynthetic steps were later characterized by Pateraki *et al.*²⁹ using a transient expression system in *Nicotiana benthamiana*. The study revealed that several enzymes from the cytochrome P450 CYP76AH subfamily are responsible for these monooxygenations, with their expression predominantly or exclusively localized to root cork cells. Specifically, three P450 enzymes (*CfCYP76AH15*, *CfCYP76AH8*, and *CfCYP76AH17*) were found to independently catalyze the conversion of 13*R*-manoyl oxide **79** to 11-oxo-13*R*-manoyl oxide **80**. Among these, *CfCYP76AH15* demonstrated the highest activity and specificity. Furthermore, engineered variants of *CfCYP76AH15* have exhibited even greater efficiency.¹⁶⁷ Subsequent monooxygenation of 11-oxo-13*R*-manoyl oxide occurs at positions C-1, C-6, and C-7, primarily mediated by *CfCYP76AH11*, leading to the production of 9-deoxy-7-deacetylforskolin **81**. Monooxygenation at position C-9, a critical step, is catalyzed exclusively by *CfCYP76AH16*, resulting in the final intermediate, 7-deacetylforskolin **82**. Collectively, these enzymes account for all oxygenated positions in forskolin.²⁹ The final step, a regiospecific acetylation of 7-deacetylforskolin, is carried out by the acetyltransferase *CfACT1-8*, yielding forskolin **17**.

3.6. Oridonin in *Isodon rubescens*

3.6.1. Occurrence and medical applications of oridonin. *Isodon rubescens* (Hemsley) has been used for a long time in traditional Chinese and Japanese medicine for its anti-inflammatory effects¹⁶⁸ that are mostly attributed to oridonin **19** (Fig. 6B). The structure and absolute configuration of oridonin was elucidated by Fujita *et al.* in 1970.¹⁶⁹ Oridonin has hydroxyl groups at positions C1, C6, and C14, and a hemiacetal bridge from C20 to C7. The keto-group at C15 and the exocyclic methylene group form an α -methylene-cyclopentanone in the D ring. Similar to many sesquiterpene lactones,²¹ oridonin

undergoes Michael addition reactions to many biological nucleophiles that, in part, explains its bioactivity. When elucidating downstream reactions in the biochemical pathway this may lead to cysteine and glutathione adducts that need to be taken into account.^{13,170} Due to a specific cytotoxicity against AML1-ETO acute myelogenous leukaemia (AML) cells a derivative of oridonin is being tested in clinical trials (HAO472, phase I, trial number: CTR20150246). Most recently, it was shown that oridonin is capable of binding to the conserved coronaviral Nsp9 SARS-CoV-2 protein, thereby inhibiting coronaviral replication with a single digit micromolar concentration.¹⁷¹

3.6.2. Oridonin biosynthesis. A first step towards the elucidation of oridonin biosynthesis was achieved in 2017 when Pelot *et al.*¹⁷² as well as Jin *et al.*¹⁷³ characterized a set of *Isodon rubescens* diterpene synthases. GGPP **8** is converted to *ent*-CPP **18** by IrCPS4* (Fig. 6B), and subsequently to *ent*-kaurene **83** by IrKSL5.^{5,173} To convert *ent*-kaurene to oridonin, oxidation reactions at six positions in the backbone are required. A huge leap forward was the identification of a gene cluster of tandem-duplicated CYP706V oxidase genes by Sun *et al.*⁵ that enabled the hydroxylation of kaurene at the positions C7, C14 and C15 (all in β -orientation).⁵ Three CYP706V enzymes (IrCYP706V2, 3, 8) independently convert *ent*-kaurene to 7 β -hydroxy-kaurene **84** and five enzymes of this subfamily (IrCYP706V4, 7, 9, 15, 16) to 15 β -hydroxy-kaurene **85**. A single P450 (IrCYP706V6) was found that produced 14 β -hydroxy-kaurene **86**. CYP706 oxidases were found to be localized in tandem repeats on chromosome 2. All these oxidation reactions of *ent*-kaurene are required *en route* to oridonin, but the order in which they take place has not been established.

3.6.3. Potential applications for metabolic engineering of oridonin production. Intriguingly, P450 enzymes from other Lamiaceae species can introduce the required additional oxidations with the correct stereochemistry, but on other backbones: CYP76AH11 from *Coleus forskholii* introduces a 1 α , 6 β and 7 β hydroxylation to manoyl-oxide (chapter 3.5) and CYP76AK (chapter 3.1) enzymes from various Lamiaceae can oxidize the C20 position in diterpene backbones.²⁹ This provides an excellent opportunity for metabolic engineering: elusive steps in a metabolic pathway could be bridged by combining enzymes from different plant species in a heterologous host such as *Nicotiana benthamiana* or yeast. Previously it was observed that CYP enzymes from the Lamiaceae and other plant families can be promiscuous towards alternative diterpene backbones while retaining their regioselectivity.¹²⁵

3.7. Scutebarbatine A in barbed skullcap (*Scutellaria barbata*)

The skullcaps (*Scutellaria* sp.) are a large genus of the Lamiaceae and are widely used in Traditional Chinese Medicine, most notably the barbed skullcap (*Scutellaria barbata*) and the Baikal skullcap (*Scutellaria baicalensis*). Scutebarbatine A **21** (Fig. 7A), from the barbed skullcap induces the apoptosis of Caco-2 cancer cells.¹⁷⁴ So far, the biosynthesis of the precursor isokolavenol **87** has been described⁷ in *S. barbata* and *S. baicalensis*. In a first step GGPP **8** is converted to IKPP **20** by SbbdiTPS2.3* in *S. barbata* and independently by SbditTPS2.7* and SbditTPS2.8* in *S. baicalensis*. In a second step IKPP **20** is converted to isokolavenol **87** by



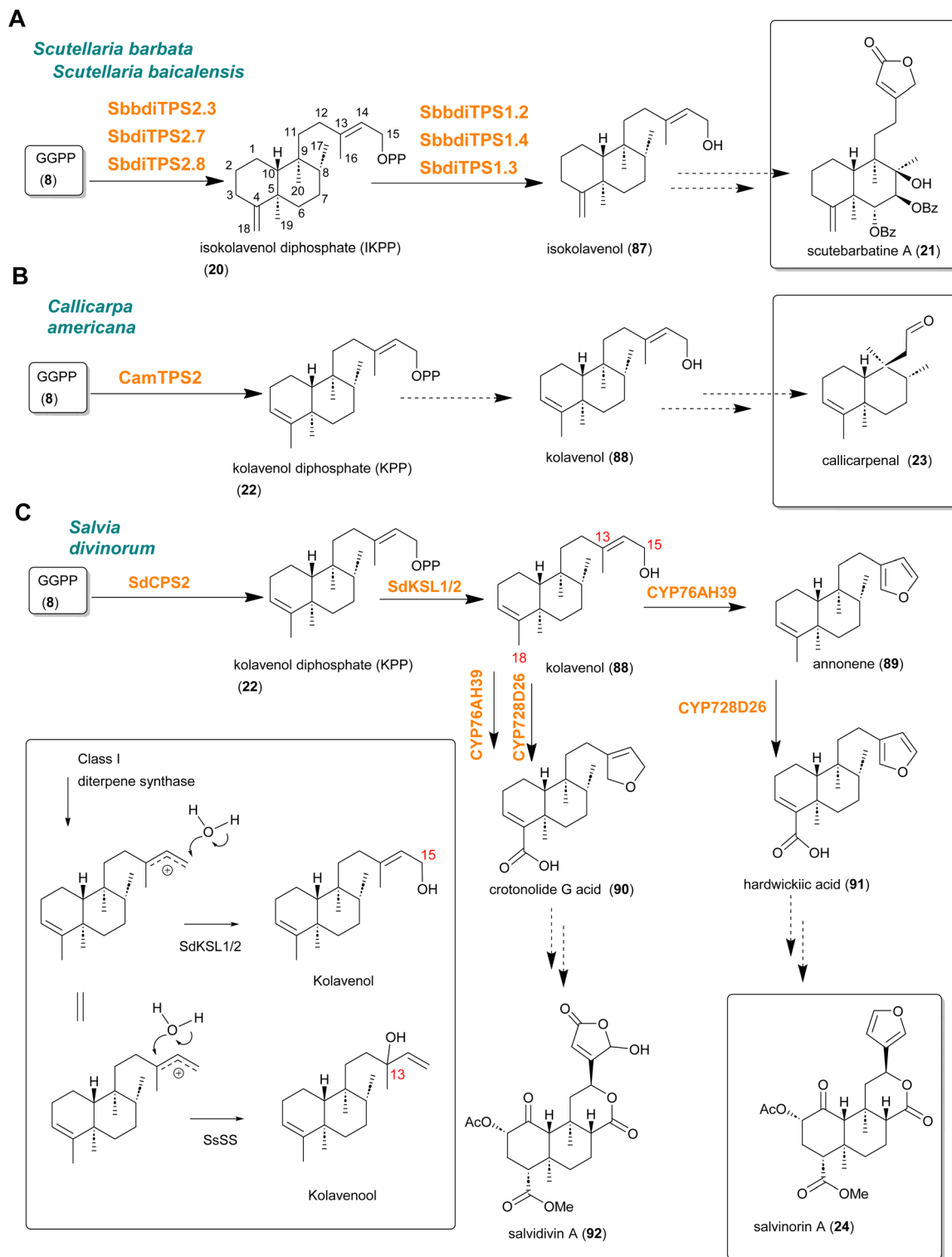


Fig. 7 Biosynthesis of (neo)clerodane diterpenes. (A) Biosynthesis of scutebarbatine A **21** in *Scutellaria* sp. (B) Biosynthesis of callicarpenal **23** in *Callicarpa americana*. (C) Biosynthesis of salvivorin A **24** in *Salvia divinorum*. CPS: copalyl pyrophosphate synthase. KSL: kaurene synthase like. SsSS: *Salvia sclarea* sclareol synthase.

SbbdiTPS1.2 or **SbbdiTPS1.4** in *S. barbata* and **SbbdiTPS1.3** *S. baicalensis*.⁷ The subsequent oxidation steps from **87** to scutebarbatine A (**21**) that include hydroxylations, esterifications to side chains and a furan ring formation have yet to be unravelled.

Four hydroxylation reactions carried out by P450 enzymes would be required for the biosynthesis of scutebarbatine A, however, no *Scutellaria* P450 gene candidate tested by Li *et al.*⁷ accepted isokolavenol as a substrate.



3.8. Callicarpenal in American beautyberry (*Callicarpa americana*)

Callicarpenal **23** (Fig. 7B), a C16 tetranor diterpene from the American beautyberry (*Callicarpa americana*), is an effective insect repellent that has been used in folk medicine. The isolation and characterization of callicarpenal were realized by Cantrell *et al.* in 2005 in a bioassay-guided fractionation approach.¹⁷⁵ A chemical synthesis was reported by Ling *et al.*¹⁷⁶ Hamilton *et al.*⁶ hypothesized that callicarpenal biosynthesis proceeds *via* kolavenol **88**. A diterpene synthase that produces KPP **22**, CamTPS2*, has been characterized. Following this hypothesis the downstream biosynthesis would involve the cleaving off a four-carbon unit, by a yet elusive mechanism. The mechanism could involve the action of a cytochrome P450 enzyme such as enzymes from the CYP87A subfamily identified in foxglove, treacle-mustard and giant milkweed that cleave the side chains of various terpenes.¹⁷⁷

3.9. Salvinorin A in divine sage (*Salvia divinorum*)

3.9.1. Occurrence and medical applications of salvinorin A.

Salvinorin A **24** (Fig. 7C) is a hallucinogenic diterpene with a furan clerodane backbone from *Salvia divinorum*. *Salvia* is the largest genus of the Lamiaceae family, yet salvinorin A is known to be synthesized in only a single species, *S. divinorum*. This hallucinogenic plant was first recorded in 1939 by an American anthropologist and linguist, Jean Basset Johnson, in Oaxacan region, Mexico, where the Mazatec people practice ritual ceremonies after chewing and inhaling *S. divinorum* leaves.¹⁷⁸ The active ingredient responsible for the hallucinogenic effect was identified as salvinorin A,^{179,180} and a comprehensive receptor-ligand analysis showed that salvinorin A is a potent and selective agonist of κ -opioid receptor with a K_i value of 4 nM.¹⁸¹

3.9.2. Salvinorin A biosynthesis. Salvinorin A **24** is a highly oxygenated clerodane diterpene bearing a furan moiety. Its biosynthesis involves many enzymatic steps, but only four enzymes have been identified in the early stage of its biosynthesis to date: clerodane synthase (SdCPS2*), kolavenol synthase (SdKSL), CYP76AH39, and CYP728D26 (Fig. 7C).^{30,182–185} The hydrocarbon backbone of salvinorin A and its derivatives is kolavenol **88**, from which distinct skeletons for salvinorin (furan), salvidivin (dihydrofuran), salvinicin (tetrahydrofuran) are framed.¹⁸² It has been proposed that kolavenol is synthesized by sequential catalysis of the class II and the class I di-TPS enzymes. The class II di-TPS (SdCPS2*), which converts GGPP **8** to KPP **22**, was identified from trichome-enriched transcriptome of *S. divinorum*.¹⁸³ Heterologous overexpression of SdCPS2* alone in tobacco and yeast is sufficient to synthesize kolavenol **88** through the cleavage of phosphate moiety by endogenous phosphatase activity.³⁰ Although the class I di-TPS enzyme can synthesize kolavenol from KPP removal of diphosphate (Fig. 7C, inset), extensive characterizations of the class I di-TPSs had difficulties in identifying the cognate di-TPS,^{183,184} but recent study demonstrated that SdKSL1/2 can catalyze the formation of kolavenol through ionization-initiated carbocation reaction.

It is noteworthy that cleaving the pyrophosphate attached to C-15 by di-TPS results in the delocalized allylic carbocation through C13 to C15 (Fig. 7C, inset). The water quench on the C-13 leads to the formation of kolavenol as shown in SsSS (class I di-TPS; sclareol synthase from *Salvia sclarea*),²⁰ while the water quench on the C-15 results in kolavenol.¹⁸⁵ Therefore, distinct class I di-TPSs are able to synthesize isomeric clerodane products with different regioselectivities. From kolavenol, CYP76AH39 catalyzes the formation of crotonolide G (dihydrofuran clerodane)¹⁸² and CYP76AH39 homolog from the ornamental sage (*Salvia splendens*) synthesizes annonene **89** (furan clerodane).¹⁸⁵ However, it remains to be investigated what sequence variations of these two enzymes in the CYP76AH subfamily lead to the formation of different products. The C-18 carbon of both crotonolide and annonene are sequentially oxygenated three-times by CYP728D26 to form crotonolide G acid **90** and hardwickiic acid **91**, respectively. Therefore, a full set of enzymes for the synthesis of salvidivin A **92** and salvinorin A **24** backbones have been elucidated in *S. divinorum*. The whole genome assembly of *S. divinorum* of 541 Mb has been recently released,¹⁸⁶ and additional enzymes (CYP, acyl transferase, and methyl transferase) for salvinorin A biosynthesis are expected to be characterized in coming years.

3.10. Other diterpene biosynthetic pathways in Lamiaceae

In a database-driven approach Johnson *et al.*¹⁸⁷ identified nine class II and ten class I diTPS across the Lamiaceae by screening 48 species across the major Lamiaceae lineages. One notable observed diTPS activity was the identification of ArTPS2 from common bugle (*Ajuga reptans*) that produced neo-clerodane-4(18),13E-dienyl pyrophosphate. This could be the entry point for the biosynthesis of bioactive epoxy-clerodane diterpenoids such as ajugarin I, clerodin and jodrellin A.¹⁸⁷ Recently, a genome assembly of teak (*Tectona grandis*) allowed the identification of terpene synthases leading to miltiradiene and *ent*-kaurene.¹⁸⁸

4. Conclusions

From ancient Chinese medicinal herbs to mediterranean spices and psychedelic compounds from Central America, the diversity of terpenes in the Lamiaceae around the world is intertwined with our history and culture. This amazing diversity of especially mono- and diterpenes is in large part based on terpene synthases and cytochrome P450 enzyme diversity. In the last two decades, a huge leap in the elucidation of the underlying biochemical pathways has been made by the scientific community. Especially in the last few years a combination of the analysis of differential transcriptomes and of biosynthetic gene clusters based on whole genome sequencing has accelerated the pathway discovery.^{8,189}

There are, however, many biochemical pathways that remain unresolved. This can be based on the complexity of the pathways or unexpected enzyme types that are involved. Some genes might be expressed only in very specific cell types and developmental stages. Here, a solution could lie in advancements in



single-cell metabolomics and transcriptomics. For many plants there is no sufficient genomic sequence information available, yet. Ambitious high-throughput genome sequencing projects like the Darwin Tree of Life Project and the Earth BioGenome Project may increase the speed of pathway elucidation and expand the chemical space accessible for metabolic engineering far beyond our current knowledge.^{190,191} For some of the most interesting compounds – in terms of biosynthesis and potential pharmaceutical applications – we show what has been achieved so far and how the road ahead may look like. The research of the future will bring us closer to an understanding of terpene biosynthesis in the Lamiaceae, enabling us to reconstruct the metabolic pathways and produce completely new terpenes *via* combinatorial biosynthesis.^{125,187}

5. Author contributions

M. F. conceptualized the manuscript. All authors were involved in the writing of the original draft as well as reviewing and editing of the manuscript.

6. Conflicts of interest

There are no conflicts of interest.

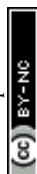
7. Data availability

All required data is available as a SI file.

SI Table 1 contains a list of CYP enzymes involved in Lamiaceae terpene metabolism. See DOI: <https://doi.org/10.1039/d5np00026b>.

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