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Frog farnesyl pyrophosphate synthases and their role as non-canonical terpene synthases for bisabolane sesquiterpenes

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Three enzymes from African frogs with close sequence homology to avian farnesyl pyrophosphate synthase (FPPS) were studied for their function. All three enzymes converted (2*Z*,6*E*)-FPP into several bisabolane sesquiterpenes, with bisabolol and anymol as main products. Experiments with FPPS from *Escherichia coli* confirmed the same function, suggesting that the observed activity may be of general relevance for FPP synthases. Only one of the frog enzymes showed significant activity in the biosynthesis of FPP from terpene monomers, which may point to an evolutionary process that resulted in a functional switch from an FPPS to a bisabolane synthase. The physiological relevance of these findings is supported by the identification of bisabolol/anymol in gland extracts.

Terpene synthases are remarkable biocatalysts that can convert structurally simple oligoprenyl pyrophosphates into highly complex often polycyclic hydrocarbons or alcohols. These transformations require just one enzymatic step, but in fact the introduced structural complexity requires a multistep cationic cascade reaction that proceeds within a hydrophobic active site cavity. This process cannot be observed directly, *e.g.* spectrometrically because of the low concentrations of the cationic intermediates in the terpene synthase mediated cyclisation reactions. Instead, indirect methods must be used such as structural characterisation of trace products that may represent deprotonation products of proposed cationic species along the cyclisation cascade,[1] isotopic labeling experiments,[2] DFT calculations,[3] structure-based site-directed mutagenesis,[4,5] and QM/MM modellings of intermediates inside an experimentally obtained enzyme structure or an AI-generated structural model.[6]

Terpene synthases have been characterised from all kingdoms of life. Some of the earliest representatives include the plant-derived humulene/caryophyllene synthase from *Salvia officinalis*,[7] (*S*)-limonene synthase from *Mentha spicata*,[8] and taxa-4,11-diene synthase from *Taxus brevifolia*[9] that produces the precursor hydrocarbon to the anticancer drug taxol.[10,11] Also from fungi, terpene synthases are long known, exemplified by trichodiene synthase from *Trichothecium roseum*[12] and from *Fusarium sporotrichioides*,[13] as well as *epi*-aristolochene synthase from *Penicillium roqueforti*[14] and from *Aspergillus terreus*.[15] Trichodiene is of importance as the parent hydrocarbon of T2-toxin and structurally related fungal toxins,[16] and *epi*-aristolochene is further converted into PR toxin.[17,18] The first characterised bacterial terpene synthases include pentalenene synthase from *Streptomyces exfoliatus*[19] and *epi*-isozizaene synthase from *Streptomyces coelicolor*.[20] Their products are the precursors to the antibiotics pentalenolactone[21] and albaflavenone.[22] More recently, terpene synthases have been discovered in social amoebae[23,24] and even in viruses.[25]

In the context of increased genome sequencing from species across the tree of life, animal-associated terpene synthases are a recently emerging field of research.[26] Several terpene synthases have been identified in corals, including synthases for klysimplexene R, cembrene A and elisabethatriene.[27,28] These compounds are the precursors to eleutherobin[29] and other eunicellane diterpenoids,[30] various oxidised cembranoids,[31] and pseudopterosins.[32] Subsequently, sponges were identified as another source of terpene synthases, confirming the animal and not its bacterial symbionts as the native producer of sponge-derived terpenoids.[33] Both in corals and in sponges the identified enzymes are most

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closely related to microbial type I terpene synthases, suggesting an ancient horizontal gene transfer between microbes and these marine animals. In addition, a bifunctional enzyme with structural similarity to canonical isoprenyl pyrophosphate synthases (IPPS) has been identified from the bark beetle *Ips pini* that first converts dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) into geranyl pyrophosphate (GPP), followed by its further transformation into the ipsdienol precursor myrcene.[34,35] A similar IPPS-like enzyme was subsequently identified from the crucifer flee beetle *Phyllotreta striolata* that converts (2*Z*,6*E*)-farnesyl pyrophosphate (FPP) into a mixture of sesquiterpenes with (6*R*,7*S*)-himachala-9,11-diene as main product.[36] More recently, several IPPS-related enzymes with terpene synthase activity have been identified from the harlequin bug *Murgantia histrionica*,[37] from the southern green stink bug *Nezara viridula*,[38] from the tea green leaf hopper *Empoasca onukii*,[39] from the butterfly *Heliconius melpomene*,[40] and from the sandfly *Lutzomyia longipalpis*.[41,42] Besides these IPPS-like terpene synthases, first canonical type I mono-, sesqui- and diterpene synthases have recently been reported from insects (Sciaridae).[43] In addition, biosynthetic genes and enzymes for iridoid monoterpenes have been identified in the chrysomelid leaf beetle *Phaedon cochleariae*[44] and in the pea aphid *Acyrtosiphon pisum*.[45] In summary, a number of IPPS-like terpene synthases and iridoid pathway enzymes have been identified in insects, and microbial-type terpene synthases were observed in marine poriferans and cnidarians, but no terpene synthases have been characterised from higher animals including chordata.

In the framework of a comprehensive survey of volatile compounds in scent glands of amphibians, the sesquiterpenes α-himachalene (**1**), 2-*epi*-β-caryophyllene (**2**) and amorph-4-en-10β-ol (**3**) have been identified in two African frog species of the family Hyperoliidae, *Hyperolius cinnamomeoventris* and *H. kivuensis*, respectively (Fig. 1).[46,47] In addition, the macrolide gephyromantolide A (**4**), a compound that may be a degraded sesquiterpenoid, was identified in *Gephyromantis boulengeri*[48,49] (family Mantellidae), while its double bond regioisomer cinnamomeoventrolide (**5**) occurs in *H. cinnamomeoventris*.[50] Also frogolide (**6**) may be of terpenoid origin[51] (frog lactones have been reviewed in ref. 52). These substances occur in gular glands (Hyperoliidae) and femoral glands (Mantellidae) of male frogs which almost certainly are related to their reproduction and probably are used to transfer species-specific cocktails of volatile pheromones to the females during mating and egg deposition.

However, the pathways involved in the biosynthesis of terpenoids from frogs, including the remarkable substances from their scent glands, remain completely unstudied. Here we report on the identification of FPP synthase (FPPS) homologs from frogs and demonstrate that these enzymes show a side activity as anymol/bisabolol synthases with the substrate (2*Z*,6*E*)-FPP.

For the identification of potential terpene synthase homologs that may be responsible for the biosynthesis of sesquiterpenes in frogs, transcriptomes of the two species *Mantidactylus betsileanus* and *Heterixalus betsileo* from tissue samples of their respective glands were sequenced and coding sequences de-novo assembled and annotated (genomes of these species were not available and their sequencing was beyond the scope of this study given the large genome sizes of amphibians). A BLAST search using the amino acid sequence of the (6*R*,7*S*)-himachala-9,11-diene synthase from *Phyllotreta striolata* as a query revealed the presence of three homologs in *M. betsileanus* (MbFPPS1 – MbFPPS3) and one additional homolog in *H. betsileo* (HbFPPS1). All four enzymes not only showed a high sequence conservation with a pairwise amino acid sequence identity of 83%, but were also closely related to the well-known FPPS from *Gallus gallus* (Fig. 2 and S1).[53] A

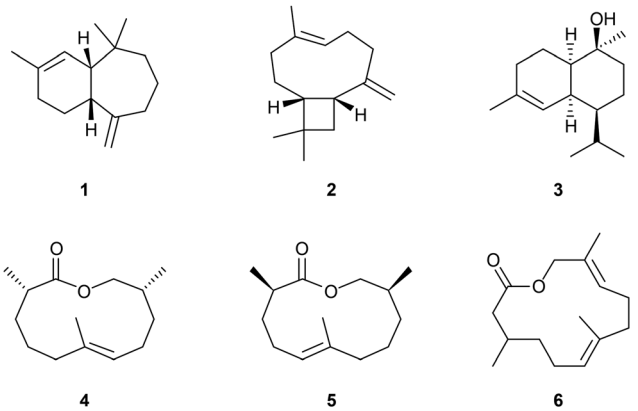


Fig. 1 Terpenoid compounds identified in the scent glands of hyperoliid and mantellid frogs.

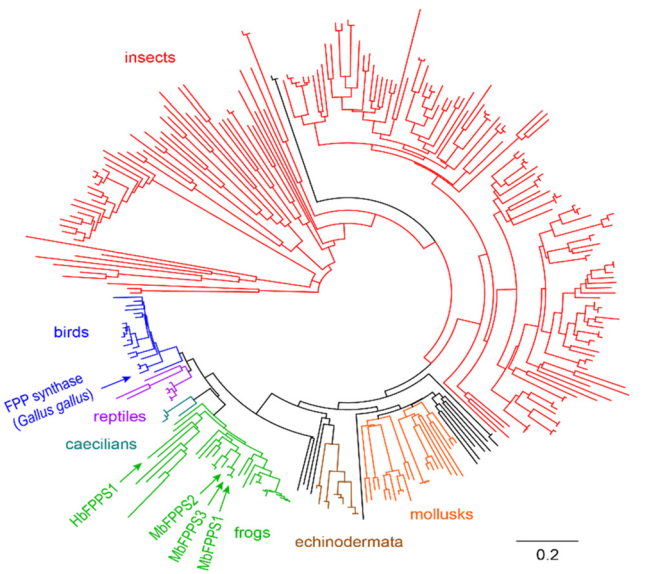


Fig. 2 Phylogenetic tree based on the amino acid sequences of the frog FPPSs characterised in this study and 298 closely related homologs from animals identified by BLAST search.

phylogenetic analysis of 299 candidate enzymes identified by a BLAST search revealed the presence of closely related homologs in many other animals including frogs, caecilians, reptiles, birds, echinodermates, mollusks and insects. Because no candidate enzymes more closely related to type I terpene synthases were found in the transcriptome data, we decided to investigate whether the discovered FPPS homologs may play a role in the biosynthesis of cyclic terpenes in frogs.

Two enzymes from *M. betsileanus* (MbFPPS1 and MbFPPS2) and the one from *H. betsileo* (HbFPPS1) were selected for functional characterisation, and their codon optimised gene sequences were cloned into the vector pYE-Express[54] through homologous recombination in yeast and expressed in *Escherichia coli* BL21 (DE3). The purified enzymes (Fig. S2) were incubated with GPP, FPP, geranylgeranyl pyrophosphate (GGPP) and geranylfarnesyl pyrophosphate (GFPP), but with all of these substrates only a formation of acyclic products known to be formed spontaneously in the presence of divalent cations (Mg^{2+} and Mn^{2+})[55] was observed (with GPP also small amounts of limonene and α-terpineol were detected; Fig. S3 and Table S2). Also the addition of other divalent cations (Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}) to incubation experiments with MbFPPS1 did not lead to any enzymatic activity. Furthermore, both pure enantiomers of the substrates linalyl pyrophosphate (LPP), nerolidyl pyrophosphate (NPP), and geranyllinalyl pyrophosphate (GLPP) were tested with MbFPPS1 and MbFPPS2, but did not result in any terpene production. Only the unusual substrate (2*Z*,6*E*)-FPP was efficiently converted by all three enzymes, and GC/MS analysis of the products pointed to the formation of a sesquiterpene alcohol as the main product, besides several sesquiterpene hydrocarbons (Fig. S4 and S5, Table S3). Despite the observation of only one peak for the main product in the GC/MS analysis, compound isolation and structure elucidation by NMR spectroscopy

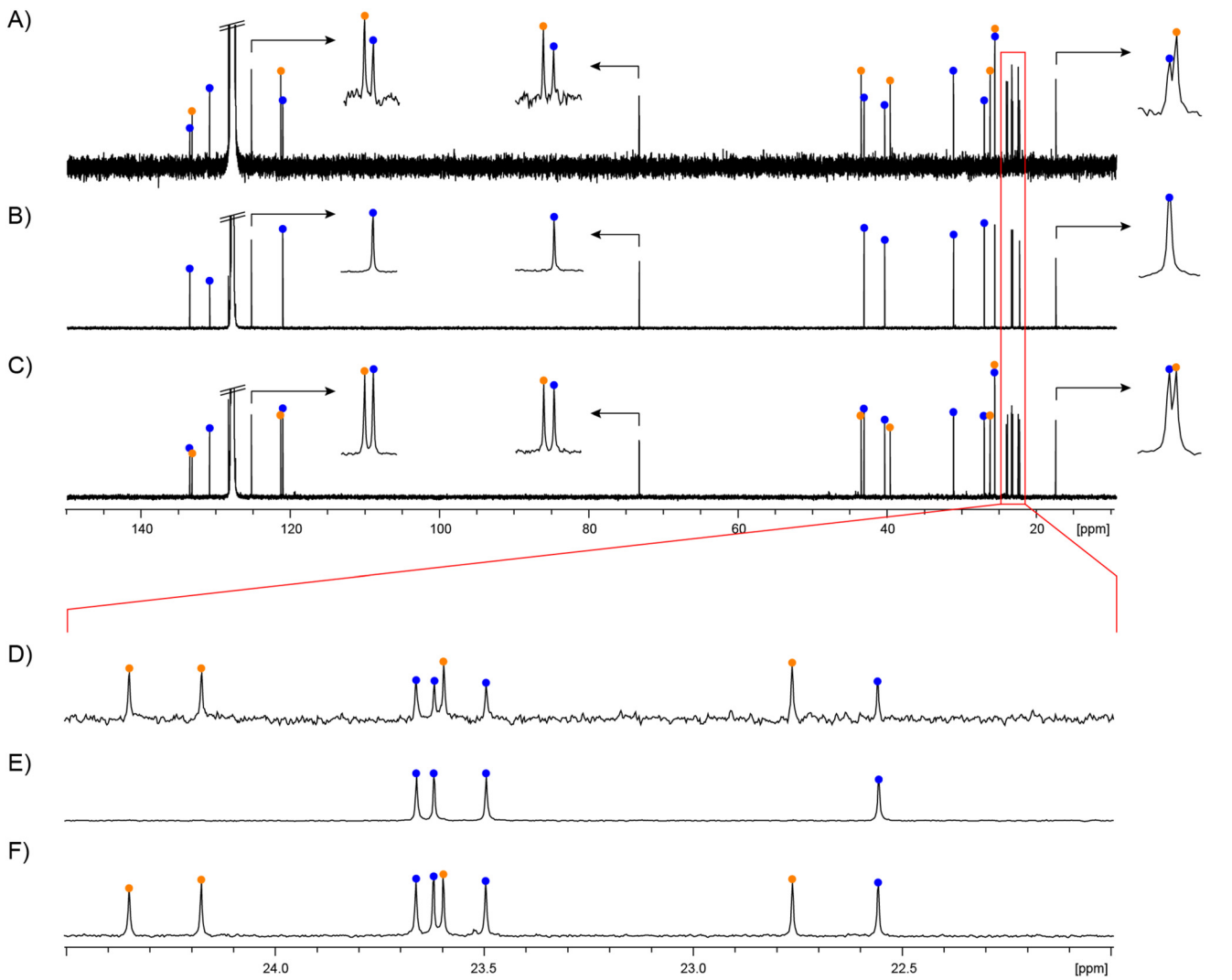
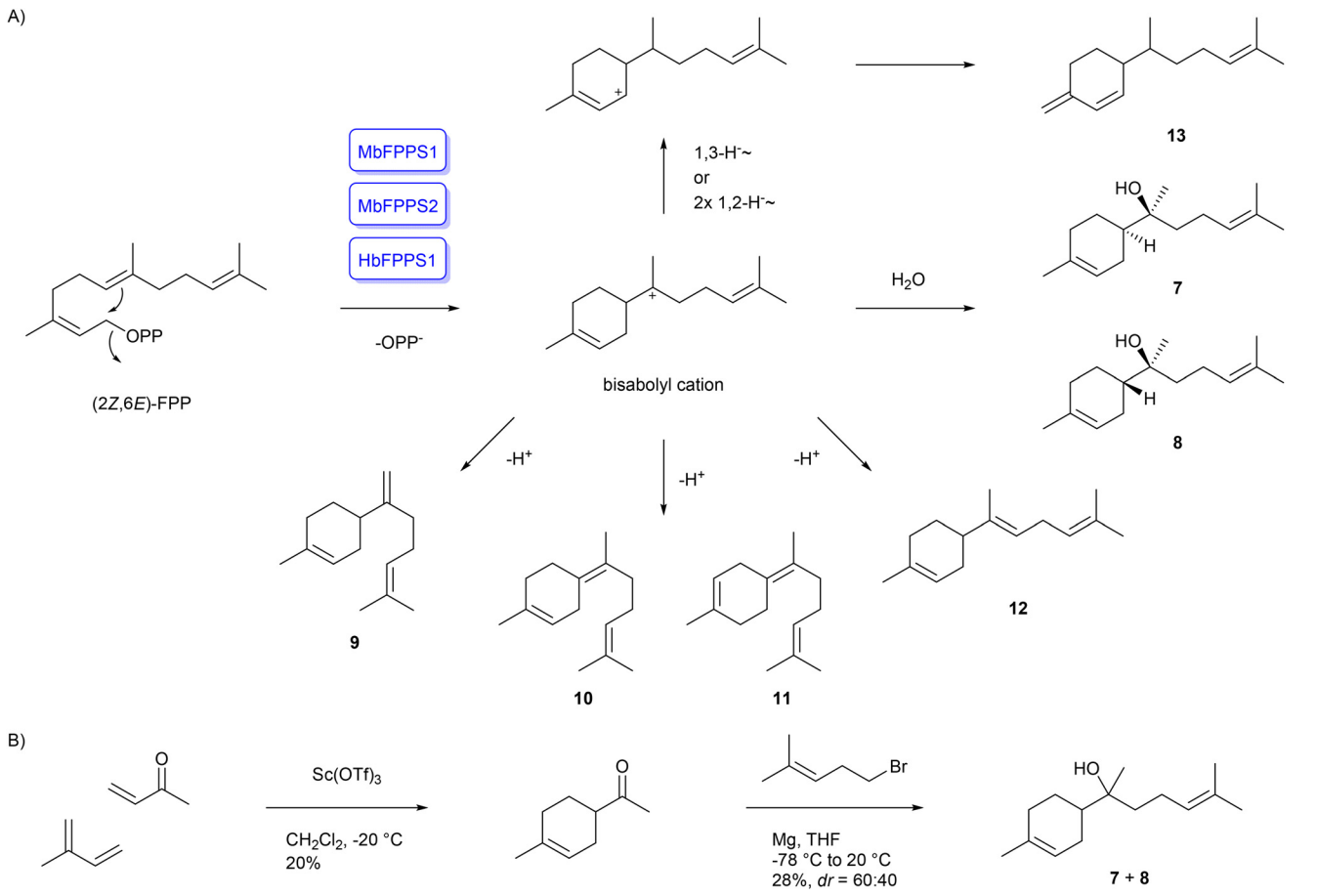


Fig. 3 Compound identification for the enzyme products anymol (**7**) and bisabolol (**8**) through ^{13}C-NMR spectroscopy. ^{13}C-NMR spectra of (A) the mixture of **7** and **8** obtained from (2*Z*,6*E*)-FPP with MbFPPS1, (B) authentic **8**, (C) the synthetic mixture of **7** and **8**. Expansions for the region between 22.0 and 24.5 ppm are shown in sections (D)–(F). The signals for **7** are marked with blue dots, signals for **8** are labelled with orange dots.

revealed the presence of two sesquiterpene alcohols in a 60 : 40 mixture that were inseparable by column chromatography on silica gel or on $AgNO_3$ impregnated silica gel. Further trials to separate the two compounds by HPLC using a variety of stationary phases were also unsuccessful. Therefore, the NMR-based structure elucidation was carried out with the compound mixture, leading to an assignment of the structures of anymol (**7**) for the major compound, and of its stereoisomer bisabolol (**8**) for the minor compound. A comparison of the ^{13}C-NMR spectrum of the compound mixture to the spectra of a commercially available authentic sample of **8** and of a synthetic mixture of **7** and **8** confirmed this structural assignment (Fig. 3 and S6, Table S4). The synthetic mixture of **7** and **8** was prepared through a $Sc(OTf)_3$ catalysed Diels–Alder reaction of isoprene and methylvinylketone, followed by addition of the Grignard reagent 4-methylpent-3-en-1-ylmagnesium bromide to the Diels–Alder adduct (Scheme 1B). The minor products obtained with both enzymes were identified through a comparison of their EI mass spectra to library spectra and of their retention indices to tabulated data from the literature as β-bisabolene (**9**), (*Z*)- and (*E*)-γ-bisabolene (**10** and **11**), (*E*)-α-bisabolene (**12**), and β-sesquiphellandrene (**13**).

Because all three frog enzymes functionally characterised in this study are closely related to avian FPPS,[56] further experiments were conducted to investigate whether the observed activity with (2*Z*,6*E*)-FPP is unique to MbFPPS1, MbFPPS2 and HbFPPS1. For this purpose, the FPPS from *Escherichia coli*[57] was incubated with (2*Z*,6*E*)-FPP, which resulted in a very similar product mixture with **7** and **8** as main products and **9–13** as side products (Fig. S4 and S5), suggesting that the observed activity may represent a second general and so far overlooked function of FPPSs. To test for their activity as FPPSs, all three frog enzymes and *E. coli* FPPS were incubated with DMAPP and IPP, followed by dephosphorylation of the products with calf intestinal phosphatase (CIP) and GC/MS analysis of the pentane extractable products (Fig. S7). Interestingly, MbFPPS1 and *E. coli* FPPS gave high yields of farnesol, while MbFPPS2 and HbFPPS1 only resulted in traces of this product. These findings suggest that MbFPPS2 and HbFPPS1 may have adapted their functions to selectively accept (2*Z*,6*E*)-FPP for the production of bisabolane-type alcohols and hydrocarbons, with no retained FPPS activity, while other FPPSs only show a side activity as terpene cyclases with the substrate (2*Z*,6*E*)-FPP.



Scheme 1 Characterisation of MbFPPS1, MbFPPS2 and HbFPPS1 from the frogs *M. betsileanus* and *H. betsileo*. (A) Cyclisation mechanism from (2*Z*,6*E*)-FPP to the main products **7** and **8** and side products **9–13**. All compounds arise through 1,6-cyclisation to the bisabolyl cation, capture with water or deprotonation, or eventually through a 1,3- or two 1,2-hydride shifts and deprotonation. (B) Synthesis of the mixture of **7** and **8**.

Conclusions

In this study we have identified a new function of three FPP synthases (FPPSs) from the frogs *Mantidactylus betsileanus* and *Heterixalus betsileo*, converting (2*Z*,6*E*)-FPP into bisabolane sesquiterpenes with bisabolol and anymol as the main products. The frog enzymes are phylogenetically related to the well characterised avian FPPS, but also the distant FPPS from *E. coli* showed the same effect. It remains to be clarified whether and to what degree this newly discovered enzyme function is physiologically relevant. So far, bisabolol/anymol among hyperoliid frogs has only been found in *Hyperolius kivuensis* (Fig. S8 and S9), but not in *Heterixalus betsileo* whose enzymes we studied herein, although other, so far unknown and partly more oxidized sesquiterpenoids were detected in both these species. Also the femoral glands of mantelline frogs, such as *Mantidactylus betsileanus*, according to available data do not contain sesquiterpenes except for the macrocyclic lactones **4–6**. At the same time, we did not find sequences coding for a putative (2*Z*,6*E*)-FPP synthase in the transcriptome assemblies of *H. betsileo* and *M. betsileanus*, which may indicate that this enzyme – required to produce the precursor that could be converted by the canonical FPPS into bisabolane sesquiterpenes – may not have been expressed in the respective samples. However, since this enzyme has so far not been detected in other frog genomes or transcriptomes, it is more likely that other gene(s) of unknown sequence have this function in frogs, or that some of the relevant compounds are taken up from the prey rather than synthetized, a possibility demonstrated for macrolides in *M. betsileanus*.[58] Comparative gene expression analysis from femoral glands *vs.* other tissues may in future studies reveal genes specifically involved in the biosynthesis or biomodification of volatile pheromone compounds, including sesquiterpenes.

Conflicts of interest

There are no conflicts to declare.

Data availability

Sequence data have been submitted to the Sequence Read archive (PRJNA1306359) and the Zenodo repository (https://doi.org/10.5281/zenodo.16877882). Other data supporting this article have been included as part of the SI, containing nucleotide and amino acid sequences of the investigated genes and enzymes including an amino acid sequence alignment, experimental methods, GC/MS analyses of enzyme products and gland extracts, and NMR data of bisabolol and anymol. See DOI: **https://doi.org/10.1039/d5ob01139f**.

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