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# Reconstructing the chemical diversity of labdane-type diterpene biosynthesis in yeast

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ABSTRACT

Terpenes are a large class of natural products, many of which are used in cosmetics, pharmaceuticals, or biofuels. However, terpene's industrial application is frequently hindered by limited availability of natural sources or low yields of chemical synthesis. In this report, we developed a modular platform based on standardized and exchangeable parts to reproduce and potentially expand the diversity of terpene structures in *Saccharomyces cerevisiae*. By combining different module-specific parts, we exploited the substrate promiscuity of class I diterpene synthases to produce an array of labdane-type scaffolds. These were subsequently modified by a scaffold decoration module consisting of a mutant library of a promiscuous cytochrome P450 to afford a range of hydroxylated diterpenes. Further P450 protein engineering yielded dedicated and efficient catalysts for specific products. Terpenes produced include precursors of pharmacologically important compounds, molecules that are difficult to obtain from natural sources, or new natural products. The approach described here provides a platform on which additional gene mining, combinatorial biosynthesis, and protein engineering efforts can be integrated to sustainably explore the terpene chemical space.


## 1. Introduction

Terpenes are an important class of natural products used as medicines, ingredients in cosmetics, neutraceuticals, or biofuels. Among them, the super-family of labdane-type diterpenes comprises over 7000 known compounds, several of which have important pharmacological properties. Tanshinones have strong anti-inflammatory activity (Robertson et al., 2014) and are active agents against various cardiovascular and cerebrovascular disorders (Cheng, 2007; Xu et al., 2011), neurodegenerative diseases (Buenafe et al., 2013; Lam et al., 2003) and cancer (Chen et al., 2012, 2013a; Luo et al., 2011; Shin et al., 2009). Forskolin activates adenylyl cyclase and increases the intracellular levels of cAMP (Daly, 1984; Seamon et al., 1981; Shoback and Brown, 1984) displaying a wide range of pharmaceutical applications (Lichey et al., 1984; Yoneyama et al., 2002). Acanthoic acid-related diterpenes have anti-inflammatory properties (Traves et al., 2014), while carnosic acid is a potent antioxidant, anti-adipogenic (Gaya et al., 2013) and anticancer agent (Danilenko et al., 2003; de la Roche et al., 2012). Other members of the group, such as sclareol and *cis*-abienol are important compounds for the fragrance industry serving as starting material for the synthesis of ambroxan, a sustainable alternative to ambergris, a fragrant compound produced by the injured intestines of sperm whales (Barrero et al., 1993; Ohloff, 1982). Labdane-type diterpenes are derived from skeletons of 20 carbon atoms that are synthesized

*Abbreviations:* TPS, terpene synthase; GPP, geranyl diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; *Ss*SCLS, *Salvia sclarea* sclareol synthase; *Cc*GGPPS, *Cistus creticus* geranylgeranyl diphosphate synthase; *Cc*CLS, *Cistus creticus* 8-hydroxycopalyl diphosphate synthase; *Sf*CDS, *Salvia fruticosa* copalyl diphosphate synthase; *Nt*ABS, *Nicotiana tabacum* abienol synthase; PaLAS, *Pichea abies* abietadiene synthase; PtAO, *Pinus taeda* abietadiene oxidase; NMR, nuclear magnetic resonance; SD medium, Synthetic Defined medium.

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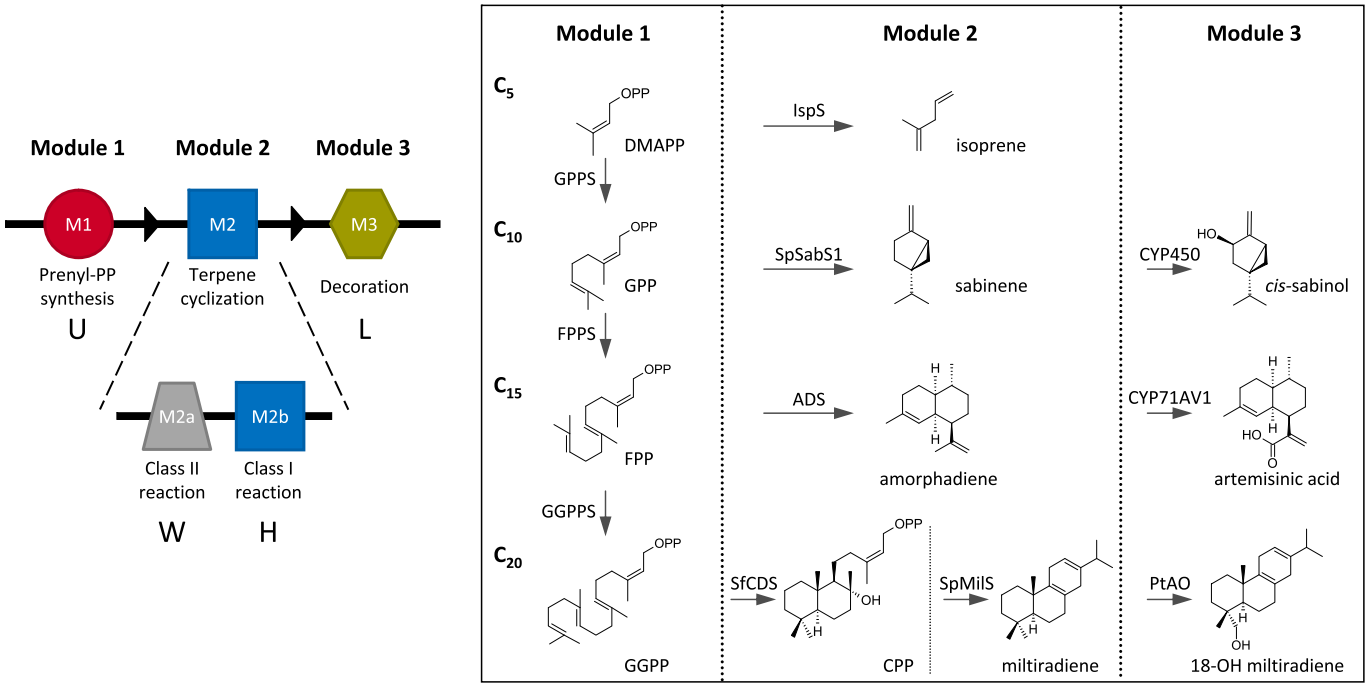


**Fig. 1.** Modular structure of terpene biosynthesis. Terpene biosynthesis can be conceptually divided into modules. Module 1 (M1) encompasses the pathway responsible for the formation of the main prenyl diphosphate substrates (DMAPP, GPP, FPP, and GGPP). The second module (M2) includes the machinery that catalyzes the formation of the basic terpene skeletons by cyclization of the different prenyl diphosphates. In labdane-type diterpene biosynthesis, M2 splits into two submodules (M2a and M2b). M2a comprises the class II diTPSs reaction responsible for the cyclization of GGPP to a diphosphate intermediate, and M2b the class I diTPSs reaction that yields the basic labdane-type structures. The third module (M3) incorporates all subsequent events resulting in the further elaboration of the labdane skeleton by modifying enzymes.

from geranylgeranyl diphosphate (GGPP) by dedicated diterpene synthases (diTPSs) (Fig. 1 and (Peters, 2010)). A typical scheme of labdane-type diterpene biosynthesis consists of an initial cyclization of GGPP by a class II diTPS to produce a cyclic diphosphate intermediate, followed by conversion of this intermediate to the final diterpene skeleton by a class I diTPS (Peters, 2010). Production of a bioactive diterpene frequently requires further elaboration of the basic structure by the addition of hydroxyl, acetyl, methyl, or sugar moieties, initiated usually by an oxygen-introducing enzyme, such as cytochrome P450s (McGarvey and Croteau, 1995; Ortiz de Montellano, 1986; Zhao et al., 2014).

Metabolic engineering has made significant progress in the heterologous production of several terpenes, with the first example being that of the anti-malarial sesquiterpene lactone artemisinin, which is now produced consistently and at a considerably lower cost than natural extractions by engineered *Saccharomyces cerevisiae* (Paddon et al., 2013; Ro et al., 2006). Other examples include the diterpenes taxadiene (Ajikumar et al., 2010; Engels et al., 2008), levopimaradiene (Leonard et al., 2010) and sclareol (Ignea et al., 2014b; Schalk et al., 2012), the sesquiterpenes $\alpha$-santalene (Chen et al., 2013b; Scalcinati et al., 2012), 5-epi-aristolochene (Nguyen et al., 2012), bisabolene (Peralta-Yahya et al., 2012), nootkatone (Wriessnegger et al., 2014), *Z,E*-farnesol (Wang et al., 2013) and *E*-$\beta$-caryophyllene (Ignea et al., 2012), the monoterpenes sabinene (Ignea et al., 2014a; Zhang et al., 2014), limonene and perillyl alcohol (Alonso-Gutierrez et al., 2013; Brennan et al., 2012), and the hemiterpene gas isoprene (Lindberg et al., 2010). However, efforts so far have mostly focused on the production of specific, industrially-relevant, terpenes. Limited emphasis has been placed on developing systems to unlock the available chemical diversity and to provide access to a range of similar, yet distinct, structures.

Here we set out to develop an approach that will facilitate recreating the diversity of terpene biosynthesis in engineered yeast cells. By applying the engineering principles of modularity and standardization, we developed a "plug-and-play" platform on which interchangeable parts can be combined to yield specific compounds. Using combinatorial biosynthesis, we revealed novel enzyme-substrate pairs, and, through protein engineering of the parts involved, we obtained new enzymatic activities and access to an array of diterpenes that are either difficult to obtain from natural sources or are new natural products.

## 2. Results and discussion

### 2.1. Exploiting the modularity of labdane type biosynthesis for the design of a "plug-and-play" platform

Conceptually, terpene biosynthesis can be broken down into modules (Fig. 1, and (Dewick, 2009; McGarvey and Croteau, 1995; Pichersky et al., 2006)). The first module (M1) consists of the machinery responsible for the formation of the universal prenyl diphosphate substrates, dimethylallyl diphosphate (DMAPP; $C_5$), geranyl diphosphate (GPP; $C_{10}$), farnesyl diphosphate (FPP; $C_{15}$), and GGPP ($C_{20}$) from IPP ($C_5$). The second module (M2) encompasses the cyclization of the prenyl diphosphate by a terpene synthase (cyclase) to yield the basic terpene structures. In the case of labdane-type diterpenes, module M2 consists of two submodules, M2a and M2b. M2a comprises the cyclization of GGPP to a labdane diphosphate intermediate by a class II diTPS, while in submodule M2b this diphosphate precursor is converted to the basic labdane-type structures by a class I diTPS. In a third (super)module, M3, these basic structures are further functionalized by modifying enzymes (cytochrome P450s, Fe(II)-dependent dioxygenases, acetyltransferases, methyltransferases, reductases, glycosyl transferases, etc.). By exploiting this conceptual modularity, we set out to reconstitute the complexity of diterpene structures using interchangeable module-specific parts. The diploid yeast strain AM102 (Mat a/α, $P_{Gal1}$-HMG2 (K6R)::HOX2, *ura3*, *trp1*, *his3*, $P_{TDH3}$-HMG2(K6R)x2-::leu2 *ERG9/erg9*, *UBC7/ubc7*, *SSM4/ssm4*) was employed as chassis. AM102 contains a