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Allison M. Heskes, Tamil C.M. Sundram, Berin A. Boughton, Niels B. Jensen, Nikolaj L. Hansen, Christoph Crocoli, Federico Cozzi, Simon Rasmussen, Britta Hamberger, Bjorn Hamberger, Dan Staerk, Birger L. Møller, Irini Pateraki

1Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark,
2Center for Synthetic Biology ‘bioSYNergy’, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark,
3VILLUM Center for Plant Plasticity, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark,
4Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia, 50728 Kuala Lumpur, Malaysia,
5Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia, 50728 Kuala Lumpur, Malaysia,
6Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia, 50728 Kuala Lumpur, Malaysia,
7Evolva A/S, Lersø Parkallé 42-44, DK-2100 Copenhagen Ø, Denmark,
8DynaMo Center, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark,
9Department of Bio and Health Informatics, Technical University of Denmark, DK-2800 Lyngby, Denmark, and
10Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2100 Copenhagen, Denmark

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SUMMARY

*Vitex agnus-castus* L. (Lamiaceae) is a medicinal plant historically used throughout the Mediterranean region to treat menstrual cycle disorders, and is still used today as a clinically effective treatment for premenstrual syndrome. The pharmaceutical activity of the plant extract is linked to its ability to lower prolactin levels. This feature has been attributed to the presence of dopaminergic diterpenoids that can bind to dopamine receptors in the pituitary gland. Phytochemical analyses of *V. agnus-castus* show that it contains an enormous array of structurally related diterpenoids and, as such, holds potential as a rich source of new dopaminergic drugs. The present work investigated the localisation and biosynthesis of diterpenoids in *V. agnus-castus*. With the assistance of matrix-assisted laser desorption ionisation-mass spectrometry imaging (MALDI-MSI), diterpenoids were localised to trichomes on the surface of fruit and leaves. Analysis of a trichome-specific transcriptome database, coupled with expression studies, identified seven candidate genes involved in diterpenoid biosynthesis: three class II diterpene synthases (diTPSs); three class I diTPSs; and a cytochrome P450 (CYP). Combinatorial assays of the diTPSs resulted in the formation of a range of different diterpenes that can account for several of the backbones of bioactive diterpenoids observed in *V. agnus-castus*. The identified CYP, *VacCYP76BK1*, was found to catalyse 16-hydroxylation of the diol-diterpene, peregri-nol, to labd-13Z-ene-9,15,16-triol when expressed in *Saccharomyces cerevisiae*. Notably, this product is a potential intermediate in the biosynthetic pathway towards bioactive furan- and lactone-containing diterpenoids that are present in this species.

Keywords: bioactive diterpenoid, *Vitex agnus-castus*, terpene synthase, cytochrome P450, MALDI-MSI imaging, Lamiaceae.

INTRODUCTION

Plants are a rich source of bioactive compounds, with numerous species used for millennia in traditional medicines. Advances in bioassay technology coupled with modern analytical techniques have now made it relatively
straightforward to identify specific bioactivities and to pinpoint the responsible active components in medicinal plants (Wubshef et al., 2016). The subsequent drug development process and (potential) wide-scale use of any identified compounds requires a reliable and scalable source of material that is both ethically and commercially viable. This requirement is often not met by the natural plant source due to difficulties with cultivation, endangered wild populations and the inherent chemical complexity found in most medicinal plant species. While chemical synthesis can potentially provide a solution to this problem, the large number of stereocenters present in natural products makes this route challenging technically and economically. Another approach to solving this problem is via the engineering of microbial organisms to produce specific natural products using biosynthetic pathways reconstructed from the original or even a combination of plant species. Examples using Saccharomyces cerevisiae as a host to make important plant-derived pharmaceuticals and nutraceuticals are already available, and include: artemisinic acid, a precursor of the anti-malarial drug artesinin, naturally produced by the plant Artemisia annua (Paddon et al., 2013); the antioxidant compound carnosic acid from Rosmarinus officinalis (Ignea et al., 2016; Scheler et al., 2016); and the cAMP booster, forskolin, from Coleus forskohlii (Pateraki et al., 2017). Through metabolic engineering of host organisms, routes to specific compounds can be optimised to increase yields and the generation of further chemical diversity explored through the application of combinatorial techniques (Ignea et al., 2015; Andersen-Ranberg et al., 2016; Jia et al., 2016). For such an approach to work, the enzymes involved in the biosynthesis of a given compound are required, and this has spurred interest in biosynthetic pathway discovery in medicinal plants.

The present work focuses on diterpenoid pathway discovery in Vitex agnus-castus L. (Lamiaceae), a shrub that grows throughout the Mediterranean region and parts of Asia. It has a long history of use in the treatment of female reproductive conditions, and is still used today to treat premenstrual syndrome (PMS). The efficacy of crude fruit extracts for relieving symptoms of PMS and related syndromes is strongly supported by evidence from multiple clinical trials (Schellenberg, 2001; Atmaca et al., 2003; He et al., 2009), and include relief from latent hyperprolactinemia and cyclic mastalgia (Kliiicag et al., 2004; Carmichael, 2008). In addition, extracts are considered to be safe and better tolerated than conventional hormone or selective serotonin reuptake inhibitort-based treatments (Daniele et al., 2005). The pharmacological properties of V. agnus-castus are attributed to the presence of dopamine, opioid and oestrogen receptor ligands that are involved in modulating hormone levels that impact PMS (Wuttke et al., 2003; Webster et al., 2011). Specifically, those compounds thought to be responsible for the observed prolactin-lowering effect are diterpenoids that interact with dopamine receptors in the pituitary gland (Hoberg et al., 1999; Jarry et al., 2006; Brattström, 2014). The ability of V. agnus-castus diterpenoids to interact with both D2 and D3 dopamine receptors in the brain has also flagged it as a potential source of new drugs that could treat other illnesses in which dopaminergic pathways play key roles (Brattström, 2014).

A large variety of labdane-related diterpenoids has been isolated from the Vitex genus, with a recent review listing 114 unique structures covering a range of classes, including labdanes, abietanes and clerodanes (Yao et al., 2016). The majority of diterpenoids isolated from V. agnus-castus incorporate hydroxyl groups at C-9 and C-13 (Figure 1a; Hoberg et al., 1999; Ono et al., 2008, 2009, 2011). Common structural features of V. agnus-castus bioactive diterpenoids are the presence of furan, lactone or 3-hydroxy-3-methyl-pent-4-enyl groups ( König, 2014). Furan or lactone groups, in particular, are frequent features of bioactive diterpenoids and appear, in some cases, to be important for their activity (Lim et al., 2012; Shul’ts et al., 2014). For example, work on elucidating the pharmacophore of salvinorin A, indicates that the furan ring influences its kappa opioid receptor agonist activity (Munro et al., 2005; Riley et al., 2014). Because of their potential pharmaceutical applications, the elucidation of biosynthetic pathways towards furan and lactone diterpenoids is of particular interest (Zerbe et al., 2014; Pelot et al., 2017).

Terpene biosynthesis is carried out in plants by a family of related enzymes known as terpene synthases (TPSs). These divide into subfamilies based on phylogenetic relationships, with some degree of functional conservation (Bohlmann et al., 1998; Chen et al., 2011). In angiosperms, the core structure of labdane-related diterpenoids is typically formed from geranylgeranyl diphosphate (GGPP) via the sequential action of two diterpene synthases (dITPS; Peters, 2010). The first enzyme, a class II dITPS (TPS-c subfamily), carries out a protonation-initiated cyclisation of GGPP to generate the characteristic decalin core. The formation of GGPP upon cyclisation – as determined by the active site of the specific class II diTPS – determines the stereochemistry of the decalin core, and this generally remains unchanged in later biosynthetic steps (Andersen-Ranberg et al., 2016). It is possible for water to capture the cyclised diphosphate carbocation resulting in the introduction of a hydroxyl group (Caniard et al., 2012; Zerbe et al., 2014). The second enzyme, a class I dITPS (usually from the TPS-e/f subfamily), then ionises the cyclic intermediate through removal of the diphosphate group and often guides further carbocation-driven rearrangements. Again, water capture of the carbocation intermediate can occur, resulting in the introduction of a (second) hydroxyl group (Caniard et al., 2012). From this scheme, the initial steps on the biosynthetic route to dopaminergic diterpenoids in
V. agnus-castus can be predicted to proceed via an initial class II diTPS-catalysed cyclisation of GGPP to generate bicyclic intermediates, potentially oxygenated at C-9 followed by rearrangement and dephosphorylation by a class I diTPS to yield either bi- or tricyclic labdane-related diterpenoids. Subsequent oxidative steps in the pathway towards the highly functionalised diterpenoids of V. agnus-castus are then likely to be carried out by members of the large cytochrome P450 (CYP) family (Ignea et al., 2016; Scheler et al., 2016; Pateraki et al., 2017).

To identify enzymes involved in the biosynthesis of V. agnus-castus diterpenoids, we first localised diterpenoids to glandular trichomes on the surface of fruit and leaves with the aid of matrix-assisted laser desorption ionisation-mass spectrometry imaging (MALDI-MSI). A leaf-trichome transcriptome database was then generated and mined for candidate diTPSs and CYPs. Functional characterisation of the identified diTPSs resulted in the discovery of two diTPS combinations capable of producing a series of C-9 hydroxylated diterpenes. A CYP with high relative expression levels in trichomes was also characterised and found to hydroxylate the diol-diterpenoid, peregrinol, to yield labd-13Z-ene-9,15,16-triol, a potential intermediate in the biosynthetic pathway towards bioactive diterpenoids with lactone and furan groups.

RESULTS AND DISCUSSION

Tissue-specific localisation of diterpenoids in Vitex agnus-castus

Terpenoids often accumulate in plant tissues at or near their site of biosynthesis. This tendency has been exploited in biosynthetic pathway discovery efforts as a way to indirectly find tissues enriched in transcripts of relevant genes that can then be targeted for transcriptomics (Trikka et al., 2015; Luo et al., 2016; Pateraki et al., 2017). Consequently, as a first step towards diterpenoid pathway discovery in V. agnus-castus, we focused our investigation on the metabolite profile and localisation of diterpenoids in fruit – the part of the plant traditionally used for treating PMS (Figure 1b). To determine if diterpenoids are localised to specific tissues within fruit, MALDI-MSI was used to map the spatial distribution of metabolites across longitudinal sections (Figure 1c). This technique has been used to localise diverse specialised metabolites in plant tissues (for a comprehensive review, see Boughton et al., 2015), including terpenoids in the medicinal plants Tripterygium wilfordii (Lange et al., 2017) and Salvia divinorum (Chen et al., 2017).

Analysis of fruit sections revealed the presence of many compounds at the outer edge of the fruit with predicted...
formulea consistent with diterpenoids previously reported from this species (Figure 1c; Table 1). Their observed distribution was found to overlap with that of glandular trichomes (Figure 1d), which were subsequently isolated, extracted and analysed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) to test for the presence of diterpenoids. Co-elution with a standard confirmed one of the major peaks to be vitexilactone (1), a labdane-type diterpenoid with a five-membered lactone ring, and of interest for its potential bioactivity (Figure 1a; Meier et al., 2000). A lack of other available reference compounds for diterpenoids demonstrated that isolated leaf trichomes and whole leaves were sequenced using Illumina HiSeq technology. Reads from the transcriptome and identification of candidate diTPSs from isolated leaf trichomes and whole leaves were sequenced using Illumina HiSeq technology. Reads from the two samples were de novo assembled using the Trinity pipeline (Haas et al., 2013). Homology searches of the transcriptome databases using characterised plant diTPSs as queries resulted in the identification of six full-length transcripts. Phylogenetic analysis of the translated coding sequences revealed that VacTPS1, VacTPS3 and VacTPS5 are members of the angiosperm class II diTPS clade assigned as TPS-c (Figure 2a; Chen et al., 2011). VacTPS1 and VacTPS3 clustered with other Lamiaceae diTPSs from specialised metabolism: VacTPS1 was most closely related to a peregrinol diphosphate synthase from M. vulgar (MvCPS1; Zerbe et al., 2014) with which it shares 61% sequence identity at the amino acid level, while VacTPS3 was placed sister to this clade. In contrast, VacTPS5 clustered in a clade of diTPSs with mixed activities, including ent-copalyl diphosphate synthases (ent-CPS) involved in the biosynthesis of gibberellins, but also several diTPSs involved in specialised metabolism. The most closely related diTPSs were an ent-CPS speculated to be involved in specialised metabolism from Isodon eriocalyx (leCPS2; Li et al., 2012) and a (−)-kolavenyl diphosphate synthase from S. divinorum (SdKPS; Chen et al., 2017; Pelot et al., 2017), both of which VacTPS5 shares 73% sequence identity at the amino acid level. The remaining three diTPSs identified from the leaf/trichome transcriptome database clustered with other class I diTPSs from the TPS-e/f sub-family (Figure 2b). VacTPS2 and VacTPS6 clustered in a Lamiaceae-specific group involved in specialised metabolism. Like other members of this group, both enzymes are missing the N-terminal γ-domain, with only the β2 bi-domain present (Hillwig et al., 2011; Pateraki et al., 2014; Zerbe et al., 2014). VacTPS4 was found to be most closely related to ent-kaurene synthases involved in gibberellin biosynthesis, sharing 78% sequence identity at the amino acid level with the closest included relative, MvEKS, from M. vulgar (Zerbe et al., 2014).

To identify terpene synthase candidates likely to be involved in trichome-specific diterpenoid metabolism, transcript levels in leaves, fruit and trichomes were quantified using quantitative real-time polymerase chain reaction (qRT-PCR). All candidates, with the exception of VacTPS4, had higher transcript levels in trichomes isolated from leaves and fruit relative to whole leaves and fruit, while VacTPS4 expression was similar across all tissues tested (Figure 2c).

Functional characterisation of VacTPSs

Characterisation of the enzymatic activity of VacTPSs was carried out using the Nicotiana benthamiana-Agrobacterium-mediated transient expression system (Bach et al., 2014). Hexane extracts of N. benthamiana leaves expressing VacTPSs were analysed by gas chromatography-mass spectrometry (GC-MS). Product identity was determined by nuclear magnetic resonance (NMR) and/or by comparison of product profiles with those of parallel assays of
Biosynthesis of Vitex agnus-castus diterpenoids

Table 1 Ions detected by MALDI-MSI found to co-localise with vitexilactone ([M + K]+ 417.2044, calculated 417.2038, 1.0 ppm error) in a fruit section of V. agnus-castus with associated calculated molecular formulae, and corresponding detection in LC-HRMS analyses of fruit and leaf trichome extracts as either [M + Na]+ or [M + H]+ ions

<table>
<thead>
<tr>
<th>Observed mass (m/z)</th>
<th>MALDI-MSI Calculated molecular formula</th>
<th>Error (ppm)</th>
<th>Adduct</th>
<th>Calculated mass for [M + Na]+ or [M + H]+</th>
<th>Observed mass (m/z)</th>
<th>Retention time (min)</th>
<th>Compounds reported from Vitex spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>345.2204</td>
<td>C_{20}H_{29}O_{2}</td>
<td>3.8</td>
<td>K+</td>
<td>329.2451</td>
<td>329.2455</td>
<td>16, 16.4</td>
<td>Sclareol (Ervig et al., 2015), viteaguzin C (Ono et al., 2008), viteaguzin D (Ono et al., 2008), peregrinol (this report)</td>
</tr>
<tr>
<td>347.2357</td>
<td>C_{20}H_{29}O_{2}</td>
<td>2.9</td>
<td>K+</td>
<td>331.2607</td>
<td>331.2612</td>
<td>16.7, 17.4, 17.8</td>
<td></td>
</tr>
<tr>
<td>359.1993</td>
<td>C_{20}H_{29}O_{3}</td>
<td>2.7</td>
<td>K+</td>
<td>343.2245</td>
<td>343.2249</td>
<td>16.15, 25.2</td>
<td>Vitexolide A (Corlay et al., 2015), viteolide B (Corlay et al., 2015), 12-epivitexolide A (Corlay et al., 2015), 12S,16S/R-di(1,3-dien)-15,16-olide (Nyligira et al., 2008)</td>
</tr>
<tr>
<td>373.1789</td>
<td>C_{20}H_{29}O_{4}</td>
<td>-3.7</td>
<td>K+</td>
<td>383.2216</td>
<td>383.2216</td>
<td>Common fragment ion</td>
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<tr>
<td>376.1943</td>
<td>C_{20}H_{29}O_{4}</td>
<td>2.9</td>
<td>K+</td>
<td>359.2193</td>
<td>359.2189</td>
<td>20.4</td>
<td>Vitexifolin F (Zheng et al., 2013), vitexifolin E (Ono et al., 2002)</td>
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<tr>
<td>383.0543</td>
<td>C_{20}H_{29}O_{3}</td>
<td>1.81</td>
<td>H+</td>
<td>405.0370</td>
<td>Not observed</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>387.2308</td>
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<td>K+</td>
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<td>371.2570</td>
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<tr>
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<td>K+</td>
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<td>401.2101</td>
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<td>385.2352</td>
<td>15.8 (F), 16.4, 17.1, 17.9, 18.5</td>
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<td>401.2306</td>
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<td>Na+</td>
<td>401.2298</td>
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<td>387.2514</td>
<td>17.5, 18.2</td>
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<td>403.0658</td>
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<td>403.0659</td>
<td>[M + H]+</td>
<td>12.6</td>
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<td>417.2044</td>
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<td>1.5</td>
<td>K+</td>
<td>401.2298</td>
<td>401.2301</td>
<td>12.6</td>
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<tr>
<td>419.2053</td>
<td>C_{18}H_{29}O_{6}</td>
<td>2.7</td>
<td>K+</td>
<td>403.2302</td>
<td>Not observed</td>
<td>13.6, 14.6 (F), 16.7</td>
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<tr>
<td>419.2201</td>
<td>C_{18}H_{29}O_{6}</td>
<td>1.56</td>
<td>K+</td>
<td>403.2455</td>
<td>403.2466</td>
<td>13.2, 13.8, 14, 15.1 (L), 16 (L), 16.8 (L)</td>
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</tr>
<tr>
<td>451.2116</td>
<td>C_{22}H_{36}O_{6}</td>
<td>0.3</td>
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<td>473.1935</td>
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<td>15.1 (L)</td>
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<td>H+</td>
<td>499.3910</td>
<td>Not observed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Co-localisation analysis was carried out using SCiLS lab software (P ≤ 0.05, threshold value of 0.5). Mass errors between observed LC-HRMS m/z values and calculated molecular formulae are < 3 ppm. Specific ions detected in only fruit or only leaf trichomes have retention times denoted by F or L, respectively, all other ions were detected in both tissues. Annotations are based on comprehensive searches of the SciFinder Scholar online chemical database for compounds with matching formulae reported from Vitex spp., with the exception of the component (m/z 401.2301) eluting at 12.6 min, which was identified as vitexilactone (1) by comparison to an authentic standard. Ions with the same mass were detected at different retention times, and may represent structural and stereoisomers and/or are the result of in-source fragmentation of different but related parent compounds. LC-HRMS, liquid chromatography-high-resolution mass spectrometry; MALDI-MSI, matrix-assisted laser desorption ionisation-mass spectrometry imaging.

Previously characterised enzymes (details are given in Table S2).

Class II diTPSs: VacTPS1, VacTPS3 and VacTPS5

Expression of VacTPS1 alone in N. benthamiana resulted in a single major peak (2; Figure 3) with a mass spectrum and retention time matching that of peregrinol (also confirmed by NMR analysis; Table S3). When coupled with MvELs, the product profile was dominated by 9,13-epoxy-14-ene (3b) as per the native M. vulgare combination of MvCPS1 and MvELs (Zerbe et al., 2014). Combined, these data indicate that VacTPS1 is a peregrinol diphosphate synthase, and is the second example of a class II diTPS that gives rise to a C-9 hydroxylated product.

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Expression of VacTPS3 in *N. benthamiana* resulted in a product profile matching that of a syn-CPS from rice, *OsCPSsyn* (Figure 4; Xu et al., 2004). Coupling of VacTPS3 with a sclareol synthase from *Salvia sclarea* (*SsSS*; Caniard et al., 2012) gave the identical product profile of *OsCPSsyn* and *SsSS* – a single major peak corresponding to syn-mano-ool/vitexifolin A (5; Andersen-Ranberg et al., 2016; Jia et al., 2016) based on retention time and mass spectrum (Figure 4). Expression of VacTPS5 in *N. benthamiana* gave a product profile matching that of TwTPS14, a kolavenyl diphosphate synthase characterised from *T. wilfordii* (Figure 5; Andersen-Ranberg et al., 2016). Correspondingly, the coupling of VacTPS5 with *SsSS* resulted in the same product profile as the characterised coupling of TwTPS14 and *SsSS*, namely the production of the clerodane-type diterpene, kolavelool (7; Andersen-Ranberg et al., 2016). To provide further evidence for the identity of the VacTPS3 and VacTPS5 products, the alcohol derivatives of the respective diphosphate products were isolated. Their structures were determined to be syn-copalol (4; Table S4) and kolavenol (6; Table S5) based on comparison of data from $^1$H NMR and heteronuclear single-quantum correlation (HSQC) experiments with data from the literature (Yee and Coates, 1992; Nakano et al., 2015), confirming that VacTPS3 is a syn-CPP synthase and VacTPS5 is a kolavenyl diphosphate synthase. To address the absolute configuration of the class II diTPS products, optical rotation measurements were attempted. Low quantities of isolated material did not allow for accurate calculation of the specific optical rotation; however, it should be noted that both 4 and 6 showed weak dextrorotatory (+1) properties. Because the absolute configurations of the class II diphosphate derivatives, 2, 4 and 6, were not established, the shown structures could be either of the two possible enantiomers for each compound, with the downstream class I diTPS products expected to share the same configuration.

**Class I diTPSs: VacTPS2, VacTPS6 and VacTPS4**

To characterise the activity of the three identified class I enzymes, all *V. agnus-castus* class II/class I enzyme
combinations were tested. Additionally, to examine the range of accepted substrates, two characterised class II diTPSs were included in the combinatorial assays: EpTPS7, an ent-CPP synthase from Euphorbia peplus (Zerbe et al., 2013); and CfTPS2, a (+)-8-LPP synthase from C. forskohii (Pateraki et al., 2014). Co-expression of VacTPS2 with VacTPS1 in N. benthamiana resulted in a shift in the VacTPS1 product profile from 2 to two main products, 3a and 8 (Figure 3). Structural analysis by NMR identified 3a as 9,13(R)-epoxy-labd-14-ene (Table S6; presumably the C-13 epimer of 3b) and 8 as viteagustinol D (Table S7). Derivatives of 3 are known from the Vitex genus (Ono et al., 2008, 2009), and 8 has been isolated from fruits of V. agnus-castus with a series of other structurally related diterpenoids (Ono et al., 2008). VacTPS2 was also found to couple with VacTPS3 to produce 5 – a diterpenoid previously isolated from Vitex rotundifolia (Figure 4; Ono et al., 2002) – as per the characterised enzyme combination of OsCPPsyn and SsSS (Andersen-Ranberg et al., 2016). A weak coupling between VacTPS2 and VacTPS5 to generate 7 was suggested by the presence of the characteristic m/z 257 and 198 ions at the correct retention time for this compound (when compared with the product profile of TtTPS14 and SsSS), but their intensity levels were too close to the background noise level for a definitive result. VacTPS2 was able to accept ent-CPP to produce ent-manool (10) and accept (+)-8-LPP to produce 13R-(-)-manool oxide (12a; Figures S1 and S2). A new product was observed when VacTPS6 was coupled with VacTPS1, which, after isolation and structural elucidation by NMR, was identified as labda-13(16),14-dien-9-ol (13; Figure 3; Table S8), a recently reported diterpenoid previously only generated by an artificial combination of a bacterial enzyme, KgTS, and MvCPS1 (Jia et al., 2016). Co-expression of VacTPS6 with VacTPS3 resulted in two main components identified as dehydroabietadiene (15) and syn-isopimara-7,15-diene (14) by comparison to the products of the characterised enzyme combination, OsCPPsyn and CfTPS3 (Andersen-Ranberg et al., 2016). VacTPS6 did not couple with VacTPS6 (Figure 5), but could accept (+)-8-LPP to produce 12a (Figure S2). VacTPS4 was not found to couple with any of the V. agnus-castus class II enzymes or CfTPS2, but when co-expressed with EpTPS7 a peak corresponding to ent-kaurene (16) was observed (Figure S3). This activity would fit with a role of VacTPS4 in gibberellin biosynthesis.

A complex array of diterpenoids with different labdane-related backbones is present in V. agnus-castus (Yao et al., 2016). The molecular basis of much of this diversity can be explained by the combined activities of the class II and class I diTPSs reported here (summarised in Figure 6). The diTPSs, VacTPS1, VacTPS2 and VacTPS6, are of particular interest as together they provide enzymatic access to C-9 hydroxylated backbones that are a feature of the bioactive preparations of this species (Jarry et al., 2006). Furthermore, VacTPS2 and VacTPS6 display a high level of...
substrate promiscuity and, as such, are valuable additions
to the combinatorial toolbox of class I diTPS capable of
catalysing the formation of valuable diterpenoids (Andersen-Ranberg et al., 2016; Jia et al., 2016). Clerodane-type
diterpenoids are also found in several Vitex species (Ono et al., 2002; Lee et al., 2013), and there is evidence that
these may also be responsible for the dopaminergic activ-
ity observed in V. agnus-castus extracts (Wuttke et al., 2003). Hence, the lack of a clear coupling between VacTPS5
and any of the tested class I diTPSs suggests there are pos-
sibly other diTPSs not characterised in this study. A ques-
tion mark also remains over the direct products of
VacTPS6 when supplied with syn-copalyl diphosphate.
Dehydroabietadiene (15) was one of two products
detected, but it is unlikely to be a direct product of
VacTPS6 due to a higher than usual level of oxidation.
Instead, 15 is likely to be the dehydrogenated derivative of
an abietane-type diterpene that has an already existing
degree of unsaturation in the C-ring. Zi and Peters (2013)
have demonstrated that miltiradiene easily undergoes
spontaneous oxidation to 15 and, as suggested by the
presence of 15 in N. benthamiana leaves and S. cerevisiae
cultures expressing a levopimaradiene synthase from
Ginkgo biloba (Matsuda and Schepmann, 2008; Brückner
and Tissier, 2013), levopimaradiene can apparently also
undergo a similarly facile aromatisation. In the present
work no such abietane-type product was detected, and so
at least one of the direct products of VacTPS6 (when cou-
pled with VacTPS3) remains to be determined.

Identification of a CYP capable of peregrinol oxidation

Formation of the furan and lactone rings characteristic of
bioactive Vitex diterpenoids is likely to be, at least in part,
catalysed by the activity of CYPs. To identify possible can-
didate enzymes for these reactions, the transcriptome data-
bases were searched for sequences with similarity to
members of the CYP76 family – a family within the large
CYP71 clan involved in the biosynthesis of specialised
diterpenoids in other Lamiaceae species (Guo et al., 2013;
Zi and Peters, 2013; Pateraki et al., 2017). A candidate gene
(VacCYP76BK1) was identified with high relative expres-
sion levels in leaf and fruit trichomes (Figure 7a). Phyloge-
netic analysis classified the candidate as the first
characterised member of a new sub-family, CYP76BK,
sister to the CYP76AK clade, which comprises CYPs involved in the biosynthesis of tanshinones and carnosic acid (Figure 7b; Guo et al., 2016; Ignea et al., 2016; Scheler et al., 2016).

The ability of the identified CYP to oxygenate the C-9 hydroxylated backbones produced by the characterised VacTPSs was tested using in vitro microsomal assays. The microsomal fraction of a S. cerevisiae strain expressing VacCYP76BK1 [along with a cytochrome P450 reductase (CPR) from poplar; Ro et al., 2002] was isolated and incubated with 2, 3a, 8 or 13. LC-HRMS analysis of assays incubated with 2 revealed the presence of a new component (17) with an [M + Na]+ ion of m/z 347.2558, which suggested the molecular formula C_{20}H_{36}O_{3} (calculated [M + Na]+ m/z 347.2557, +0.04 ppm difference; Figure 7c). No new components were observed in assays incubated with 3a, 8 or 13 compared with control assays (Figure S4a).

To confirm the activity of VacCYP76BK1 in vivo and to obtain sufficient amounts of 17 for structural elucidation, VacTPS1, SeSS (previously shown in Jia et al., 2016 to catalyse the formation of 8 from peregrinol diphasphate), VacCYP76BK1 and a CPR from C. forskohlii (Pateraki et al., 2017) were integrated into the genome of S. cerevisiae (Jensen et al., 2013). Analysis of culture extracts by LC-HRMS showed production of 2, 8 and 17 (along with additional minor components only observed when VacCYP76BK1 was present; Figure 7c). 17 was isolated from an ethyl acetate extract of an engineered S. cerevisiae culture using a combination of silica chromatography and reversed-phase-thin-layer chromatography (RP-TLC). The structure of 17 was determined by NMR analysis to be labd-13Z-ene-9,15,16-triol (Table S10), a known diterpenoid isolated from another species belonging to the Lamiaceae family, Leucas stelligera (Kulkarni et al., 2013). 17 was also found in the present study in fruit and leaf trichome extracts, along with 2, 8 and an unidentified diterpenoid produced by S. cerevisiae strains expressing the diTPSs (Figure S4b and c).

That 2 appears to be a substrate for VacCYP76BK1 is interesting considering it is the simple dephosphorylated form of peregrinol diphasphate, the product of the class II enzyme, VacTPS1. From our experimental data it could not be determined if either of the characterised class I diTPSs are involved in its formation. 2 was observed in extracts of N. benthamiana leaves transiently expressing VacTPS1 both with and without co-expression of class I diTPSs. The non-quantitative nature of the expression system made it difficult to determine whether either VacTPS2 or VacTPS6 possesses simple phosphatase activity (alongside their other characterised activities) or whether the presence of 2 in these assays was due to the activity of endogenous phosphatases in N. benthamiana, an activity previously observed in this expression system (Andersen-Ranberg et al., 2016).

Without direct evidence for the function of VacCYP76BK1 in V. agnus-castus, we can only speculate that C-16...
Figure 6. Summary of identified *Vitex* agnus-castus diterpene synthase (dTPS) activities.
(a) Observed enzymatic activities of *V. agnus-castus* dTPSs. Dashed arrow indicates 15 may not be the direct product of VacTPS6.
(b) Examples of functionalised diterpenoids from *Vitex* spp. representing the different diterpene backbones generated by the class II and class I dTPS combinations. [Colour figure can be viewed at wileyonlinelibrary.com].
hydroxylation of 2 is a native function of the enzyme in planta. Nevertheless, VacCYP76BK1 was found to be expressed in the same tissue where 2 was detected, indicating that this diterpenoid would likely be available as a substrate. The addition of a hydroxyl group at this specific position of 2 to yield 17 could be considered a step towards the formation of both the lactone and furan rings characteristic of diterpenoids present in V. agnus-castus (Figure 1a). Further oxidation at either C-15 or C-16 of 17 to form a carboxylic acid has the potential to lead to formation of a lactone by spontaneous cyclisation (Figure 8). A similar pathway has been demonstrated for the biosynthesis of costunolide – a sesquiterpene lactone found in several Asteraceae species. Here, the C-13 methyl of the sesquiterpene backbone, germacrene A, is successively oxidised to a carboxylic acid via an aldehyde by a single CYP (de Kraker et al., 2001). Hydroxylation of the nearby C-6 by a second CYP then leads to spontaneous cyclisation, resulting in lactone formation (de Kraker et al., 2002; Liu et al., 2011). In the case of artemisinic acid biosynthesis, an alcohol dehydrogenase and an aldehyde dehydrogenase are responsible for catalysing these steps from the sesquiterpene alcohol to the acid (Teoh et al., 2009; Paddon et al., 2013). A route to furan ring formation from 17 is also feasible via the aldehyde intermediate involved in lactone ring formation. In this scheme, tautomerisation of the aldehyde in a keto-enol equilibrium reaction followed by simple dehydration would afford the furan ring (Figure 8). Alternative routes to furan formation have been proposed, and involve the formation and subsequent loss of a 9,13-epoxy bridge in conjunction with an additional oxidation of C-15 or C-16 (Henderson and McCrindle, 1969; Zerbe et al., 2014). It seems, however, that the presence of the C-9 hydroxyl (and by extension the 9,13-epoxy bridge) is not essential for furan or lactone ring formation as multiple furan- and lactone-bearing diterpenoids without C-9 hydroxylation exist (Smith et al., 1982; Omosa et al., 2014; Anwar et al., 2017). Nevertheless, a potential intermediate of this alternative route was identified in the present study as a major product of VacTPS2 when coupled with VacTPS1 (3a), and similar compounds are often encountered in other furan/lactone diterpenoid-producing species (Ono et al., 1999; Tesso and König, 2004; Kulkarni et al., 2013). The sheer number of structurally related diterpenoids

Figure 7. Phylogenetic analysis, relative transcript abundance in Vitex agnus-castus tissues and activity of VacCYP76BK1.
(a) Relative transcript abundance of VacCYP76BK1 in leaves, fruit and trichomes as determined by quantitative real-time polymerase chain reaction (qRT-PCR). For leaf data, the mean of three biological replicates (± 1 SD) is shown; for fruit, tissue was combined from three different plants to obtain sufficient trichome RNA for expression studies. Two-tailed P-values < 0.05 are shown with an asterisk.
(b) Maximum Likelihood tree of cytochrome P450s (CYPs) based on aligned protein sequences. The tree is drawn to scale, with branch lengths representing substitutions per site. Branches with > 80% bootstrap support are indicated with a filled circle. The tree was rooted with MsCYP71D18. Genbank accession numbers are listed in Table S9.
(c) Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) total ion chromatograms of microsomal assay extracts prepared from a Saccharomyces cerevisiae strain expressing VacCYP76BK1 supplied with peregrinol (2), and culture extracts of S. cerevisiae strains expressing either diterpene synthases (diTPSs) alone or in combination with VacCYP76BK1.
found in *V. agnus-castus* points to the existence of a complex metabolic grid comprised of (multifunctional) enzymes, which combine to generate the observed chemical diversity. In such a system, multiple routes to furan and lactone diterpenoids may exist.

In the present work, we have described the functional characterisation of a family of diTPSs in *V. agnus-castus*. Combined, the diTPSs could generate a range of diterpene backbones that form the biosynthetic basis for the multitude of highly functionalised labdane- and clerodane-type diterpenoids observed in this species. Several of these functionalised diterpenoids possess unique therapeutic properties, and the described CYP offers a potential enzymatic step towards their biosynthesis. Our findings accordingly show how *V. agnus-castus* offers a valuable source of enzymes that can be implemented in the engineering of biosynthetic pathways for the production of high-value diterpenoids.

**EXPERIMENTAL PROCEDURES**

**Plant material**

*Vitex agnus-castus* seeds were obtained from a plant growing in a private garden in Chania (Crete, Greece), and were germinated and grown in the greenhouse at the University of Copenhagen, Denmark (minimum day length/temperature: 11 h/12°C). Samples of leaves and semi-mature fruit were harvested from three individual plants. Tissues were ground in liquid nitrogen and stored at −80°C until use or prepared for MALDI-MSI (see below).

**MALDI-MSI and sample preparation**

*Vitex agnus-castus* fruit were embedded in denatured albumin (boiled egg-white) and frozen over a bath of liquid nitrogen. Albumin embedded fruit were cryosectioned (Leica CM3050 S cryostat, Leica Microsystems, Wetzler, Germany) and sections gently pressed onto pre-chilled double-sided carbon tape attached to glass slides. Slides were freeze-dried overnight and stored in a vacuum desiccator. 2,5-Dihydroxybenzoic acid was used as matrix and sublimed onto tissue sections using a custom-built sublimation apparatus at temperatures of 130–140°C at vacuum pressures of <0.1 mbar for a period of 4–6 min. A Bruker SolariX 7 XR Tesla Hybrid ESI/MALDI-FT-ICR-MS (Bruker Daltonik, Bremen, Germany) was used to acquire MS images. For data acquisition and analysis details, see Data S1.

**Metabolite analysis**

For metabolite analyses, ground *V. agnus-castus* tissues were extracted with n-hexane or acetonitrile with shaking incubation at 25°C for 1 h. Hexane samples were analysed by GC-MS using a Shimadzu GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) with an HP-SMS UI column (20 m × 0.18 mm i.d., 0.25 μm film thickness; Agilent, Santa Clara, CA, USA) with H2 as the carrier gas. The injection port was operated in splitless mode at 250°C and the GC
program was as follows: 60°C for 1 min, ramp to 150°C at 30°C min⁻¹, ramp to 250°C at 15°C min⁻¹, ramp to 290°C at 30°C min⁻¹; hold for 3 min. The MS used electron impact (EI) ionisation with the ion source voltage and temperature set to 70 eV and 300°C, respectively. The programmed temperature vapourisation method (PTV) was used to analyse VacTPS1 products due to known degradation issues with peregirnol-type diterpenoids (Zerbe et al., 2014). Here initial injection port temperature was held at 40°C for 1 min, then ramped to 250°C over 4 min. LC-HRMS analyses of acetonitrile extracts were performed using the coupled Ultimate 3000 UHPLC: Focused system ( Dionex, Sunnyvale, CA, USA) and Bruker Compact ESI-QTOF-MS (Bruker) system described in (Tateraki et al., 2017) with the following gradient method: 0–1 min, 20% B; 1–23 min, 20–100% B; 23–25 min, 100%; 25–25.5 min, 100–120%; 25.5–30.5 min, 20% B. Mass spectra were acquired in positive ion mode with the following ESI settings: capillary voltage, 4000 V; end plate offset, −500 V; dry gas temperature, 220°C; dry gas flow of 8 L min⁻¹; nebuliser pressure, 2 bar. Data were analysed using DataAnalysis 4.1 (Bruker).

**Quantification of vitexilactone**

Quantification of vitexilactone was carried out on diluted acetonitrile extracts by LC-MS/MS using an external standard curve of vitexilactone. For details see, Data S1.

**Transcriptome sequencing and assembly**

_Vitex agnus-castus_ leaf total RNA was extracted from expanding leaves using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St Louis, MO, USA). RNA was also extracted from a trichome-enriched leaf tissue sample achieved by shaking leaves in a dry-ice-filled 50-ml tube. Trichomes adhered to the tube walls along with some broken leaf material were washed off with lysis buffer and extracted as above. Transcriptome sequencing was carried out by Macrogen (Seoul, South Korea) on an Illuimina HiSeq2000 sequencer (Illuimina, San Diego, CA, USA) to generate paired-end libraries (2 x 100 bp). The reads were de novo assembled and relative transcript abundance estimated using the Trinity pipeline (Haas et al., 2013).

**Phylogenetic analysis**

Amino acid sequences from _V. agnus-castus_ were aligned in MEGA 6.06 (Hail, 2013) with functionally characterised diTPS or CYP amino acid sequences from other plants species retrieved from GenBank (http://www.ncbi.nlm.nih.gov/; accession numbers are given in Tables S1 and S9) using MUSCLE with default settings (Edgar, 2004). The alignments were manually trimmed to remove terminal gaps and, in the case of diTPS alignments, N-terminal plastid targeting peptides were also removed. Phylogenetic trees were constructed using the Maximum Likelihood method based on the JTT matrix-based model with a site coverage cut-off of 90%. To model evolutionary rate differences among sites, a discrete Gamma distribution was used with five categories. Trees are drawn to scale, with branch lengths representing the number of substitutions per site. Trees were tested by bootstrapping with 1000 repetitions. The class I and class II diTPS trees were rooted with PpCPS/EKS from _Physcomitrella patens_, and the CYP tree was rooted with CYP71D18 from _Mentha spicata_.

**RNA extraction and qRT-PCR**

RNA was extracted from whole leaves, fruit and trichomes (isolated by brushing the surface of frozen tissue samples) using the Ambion RNAqueous-Micro Total RNA Isolation Kit with Plant RNA isolation Aid (Thermo Fisher Scientific, Waltham, MA, USA) and DNAse treated using the Turbo DNA-free Kit (Thermo Fisher Scientific). Quality control of RNA samples was performed with a 2100 Bioanalyzer Instrument (Agilent). cDNA libraries were generated using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). qRT-PCR reactions were performed with gene-specific primers (Table S11) and the SensiFAST SYBR® ROX Kit master mix (Bioline Reagents, UK) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the following two-step cycling parameters: 95°C for 2 min, followed by 45 cycles of 95°C for 5 sec and then 60°C for 30 sec. Melt curve analysis was performed by increasing the temperature from 65°C to 95°C in 0.5°C increments. RNA polymerase II subunit 3 was used as the reference gene. Gene expression levels were calculated using the efficiency corrected delta Ct (E-A-E) method with the specific efficiency (E) of each primer pair calculated using a standard curve based on four log dilutions of cDNA template. All reactions were carried out in three technical replicates, and values reported for leaves and leaf trichomes are the mean of three biological replicates. For fruit trichomes tissue was pooled from three different plants to obtain sufficient trichome RNA for expression studies. A Student’s t-test was used to test for statistically significant differences in gene expression levels between leaves and leaf trichomes using SigmaPlot 13.0 (Systat Software, San Jose, CA, USA).

**Cloning and transient expression of candidate genes from Vitex agnus-castus in Nicotiana benthamiana**

Candidates were amplified with gene-specific primers (Table S11) from cDNA generated from leaf trichome total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The cloning procedure and transient expression of TPSs in _N. benthamiana_ was performed as described in Andersen-Ranberg et al. (2016). Five-seven days after infiltration, leaf discs were extracted and analysed by GC-MS as per ‘Metabolite analysis’. Characterised diTPSs used in the reference assays were provided by Johan Andersen-Ranberg (University of Copenhagen) in pCAMBIA13003Ssu.

**Microsome preparation and in vitro assays**

The _S. cerevisiae_ strain AM94 (Ignea et al., 2012) was transformed with the expression vectors pXY143 carrying the poplar CYP reductase, CPR2 (Ro et al., 2002), under the control of the ADH1 promoter and either PyeOp60 carrying VacCYP76BK1 under the control of the GAL10-CYC1 promoter or without the insert (empty vector) as control. Transformants were grown for 24 h at 30°C and 110 rpm in 50 ml of synthetic complete (SC) selection media (minus leucine and uracil) with 2% glucose. The cells were pelleted and resuspended in 200 ml SC selection media with 2% galactose and grown for a further 24 h. Microsomes were prepared as described in Pompon et al. (1996), and in vitro assays performed as described in Hamann and Muller (2007).

**Saccharomyces cerevisiae strain construction and testing**

The _S. cerevisiae_ strains were constructed and grown for metabolite analysis as described in Andersen-Ranberg et al. (2016). Codon optimised VacTPS1 and SsSS (GeneArt, Thermo Fisher Scientific) were used to generate 2 and also vitexagnusin D (B) (Jia et al., 2016), and were cloned into _S. cerevisiae_ genome integration plasmids for integration into the XI-5 locus along with codon optimised VacCYP76BK1 and CYP (Pateraki et al., 2017). VacTPS1 and SsSS (Caniard et al., 2012) were under the control of...
the TEF1 and PKG1 promoters, respectively, VacCYP76BK1 was under the control of the TDH3 promoter, and CCPR was under the control of the TEF2 promoter. For metabolite analysis of transformants, culture aliquots of 200 μl were extracted with an equal volume of ethyl acetate (EtOAc), vortexed and centrifuged. The EtOAc layer was dried under N2, re-suspended in 150 μl acetoni-trile and analysed by LC-HRMS as per ‘Metabolite analysis’.

**Compound isolation and NMR experiments**

For details, see Data S1.

**ACCESSION NUMBERS**

Nucleotide sequences reported in this study were submitted to the National Center for Biotechnology Information (NCBI) GenBankTM/EBI Data Bank with accession numbers: VacTSP1, MG696748; VacTSP2, MG696749; VacTSP3, MG696750; VacTSP4, MG696751; VacTSP5, MG696752; VacTSP6, MG696753; VacCYP76BK1, MG696754.

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**REFERENCES**


