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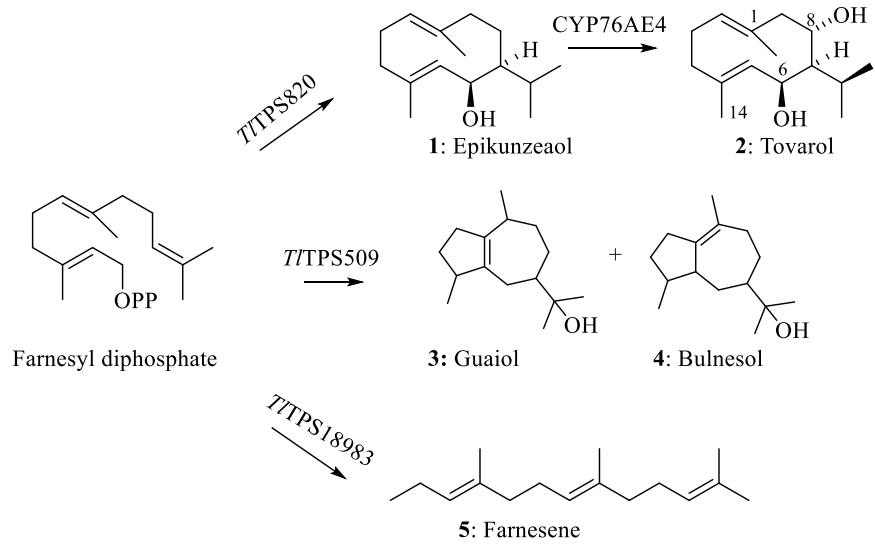
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1 Graphical abstract

2 The biosynthesis of sesquiterpenoids in *Thapsia laciniata* Rouy was investigated, and three terpene
3 synthases was characterized.



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7 **Biosynthesis of tovarol and other sesquiterpenoids in**
8 ***Thapsia laciniata* Rouy**

9

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20

21 **Abstract**

22 The genus *Thapsia* produces a wide variety of sesquiterpenoids. The Mediterranean plant *Thapsia laciniata*
23 Rouy is known to have a product profile that differs from several other species in the genus. Thus, the
24 biosynthesis of sesquiterpenoids in *Thapsia laciniata* Rouy was investigated. Here we describe three terpene
25 synthases, *TTPS820*, *TTPS509* and *TTPS18983*. *TTPS18983* is a multi-product enzyme with farnesene as
26 the major product, while *TTPS509* produces guaiol and bulnesol along with other major and several minor
27 unknown products. *TTPS820* is orthologous to *TgTPS2* from *Thapsia garganica* L. and is an epikunzeaol
28 synthase. *TgCYP76AE2* from *Thapsia garganica* performs a triple hydroxylation of epikunzeaol at C-13 to
29 make dihydrocostunolide. It was therefore investigated if the cytochrome P450, *TlCYP76AE4* was able to use
30 epikunzeaol as a substrate. It was found that *TlCYP76AE4* hydroxylates epikunzeaol at C-8 to yield tovarol
31 instead of dihydrocostunolide.

32 **Keywords**

33 *Thapsia laciniata*, Apiaceae, sesquiterpenoid, CYP76AE4, epikunzeaol, tovarol, guaiol, bulnesol

34

35 1. Introduction

36 The Apiaceae family is known to include species of great nutritional and pharmacological value, such as
37 *Daucus carota* L. (carrot) and *Thapsia garganica* L. (deadly carrot). Species from the genus *Thapsia* have
38 been used in traditional medicine for millennia for a variety of ailments including rheumatism, insect bites,
39 acne, colds and for purging (Andersen et al., 2015b). The presence of biologically active compounds, several
40 of which are sesquiterpenoids, may justify the traditional use (Simonsen et al., 2013). While many species in
41 Apiaceae have been studied for their chemical composition, only a few genes involved in biosynthesis of
42 sesquiterpenoids have been characterized. The initial enzymes involved in the specialized biosynthesis of
43 sesquiterpenoids are the sesquiterpene synthases (sqTPS). A few sqTPSs have been characterized in Apiaceae,
44 these include a sqTPS from *D. carota* producing (*E*)- β -caryophyllene and α -humulene (Yahyaa et al., 2015),
45 a δ -cadinene synthase (*Tg*TPS1) and a epikunzeaol synthase (*Tg*TPS2) from *T. garganica* (Pickel et al., 2012)
46 and the germacrene D synthase from *Thapsia laciniata* (Andersen et al., 2015a). Of these, the epikunzeaol
47 synthase *Tg*TPS2 from *T. garganica* has been of particular interest. The structure of epikunzeaol makes this a
48 likely precursor for thapsigargin. Thapsigargin is a potent inhibitor of the mammalian sarco-endoplasmic
49 reticulum Ca²⁺-ATPase (Thastrup et al., 1990) with a complex structure that requires several unknown
50 biosynthetic steps. The recently discovered cytochrome P450, *Tg*CYP76AE2, from *T. garganica* was found to
51 make a triple hydroxylation of epikunzeaol, thereby oxidizing C-13 to a carboxylic acid, which enabled
52 formation of the lactone ring present in thapsigargin (Andersen et al., 2017). While several species in the
53 *Thapsia* genus produce sesquiterpene lactones, these specific type of compounds are not found in *T. laciniata*
54 (Drew et al., 2012).

55 The *Thapsia* genus is still in need of revision and several species are unresolved. *T. laciniata* Rouy has also
56 been known as *Thapsia villosa* type II L. and is still listed as unresolved on <http://www.theplantlist.org/>.
57 Studies of marker sequences in the *Thapsia* genus, however, support the assignment of *T. laciniata* as an
58 independent species that is distinct from the classic *T. villosa* (Weitzel et al., 2014). *T. laciniata* is commonly
59 found in the southern parts of France and in Catalonia, Spain. A variety of monoterpenoids and
60 sesquiterpenoids have been reported from this species, with guaiols and germacrenols as the major
61 sesquiterpenoids in the roots (Drew et al., 2012). In *T. laciniata* the only enzyme described so far is the
62 germacrene D synthase, *TITPS*7414 (Andersen et al., 2015a). Here, we present the finding of an orthologue of
63 *Tg*TPS2 namely *TITPS*820 that also produces epikunzeaol. Two additional sqTPSs, *TITPS*18983 and
64 *TITPS*509 give further insight into the diverse sesquiterpenoid profile in *T. laciniata*. In *T. laciniata* there is
65 no equivalent sequence to *Tg*CYP76AE2, which explains the lack of sesquiterpene lactones. The orthologue
66 *TICYP*76AE4 instead catalyzes the hydroxylation of epikunzeaol at C-8 to yield tovarol.

67 2. Results

68 2.1. Identification of genes

69 A homology-based search using BLAST was initiated to investigate if *T. garganica* and *T. laciniata* harbored
70 orthologue genes for sesquiterpenoid biosynthesis. *TgTPS1* and *TgTPS2* from *T. garganica* were used as baits
71 in the *T. laciniata* Illumina transcriptome dataset (Drew et al., 2013). For *TgTPS2*, a gene with 97.2%
72 nucleotide identity was discovered. The homology search for *TgTPS1* did not yield any hits over 90% in
73 sequence identity. Three additional sqTPSs were found; the germacrene D synthase, *TITPS7414* (Andersen et
74 al., 2015a) and the unknown *TITPS509* and *TITPS18983* (Drew et al., 2013). The data set was also mined for
75 cytochrome P450s with high sequence similarity to *TgCYP76AE2*, which is the gene responsible for
76 sesquiterpene lactone formation in *T. garganica*. One gene was found with 92% amino acid sequence identity
77 and 96% nucleotide sequence identity to *TgCYP76AE2*. The gene was named *TICYP76AE4* (Nelson, 2009).
78 All genes were cloned into the pEAQ vector system for transformation into *A. tumefaciens* (Peyret and
79 Lomonosoff, 2013). *Nicotiana benthamiana* was used as an expression host to examine *TITPS820*,
80 *TITPS18983*, *TITPS509* and *TICYP76AE4*. Samples were extracted with either hexane or by HS-SPME
81 depending of the volatility of the enzymatic products.

82 2.2. Characterization of *TITPS820* expressed in *N. benthamiana*

83 Leaves from *N. benthamiana* expressing *TITPS820* were extracted with hexane and analyzed by GC-MS. The
84 analysis yielded a sesquiterpenoid profile identical to *TgTPS2* (Figure 1). The main product of *TgTPS2* is
85 epikunzeaol (**1**) (Figure 2), thus *TITPS820* is also an epikunzeaol synthase and an orthologue of *TgTPS2*. As
86 described previously, the high injection port temperature at 250°C lead to cope-rearrangement of **1** (Andersen
87 et al., 2017). Thus, **1** was partly degraded to two unknown alcohols.

88 2.3. Characterization of *TITPS509* expressed in *N. benthamiana*

89 HS-SPME-GC-MS analysis of *N. benthamiana* leaves expressing *TITPS509* resulted in the detection of two
90 major sesquiterpene alcohols, guaiol (**3**) (RI: 1585, 34% of the area) and bulnesol (**4**) (RI: 1654, 20% of the
91 area) (Figure 3). Compound **3** and **4** exhibited similar EI MS spectrum to those of bulnesol and guaiol in NIST
92 and Wiley GC-MS spectrum libraries. The product profile showed several additional compounds, and we could
93 identify two minor products; guaiene (RI 1418, Rt: 8.6)) and bulnesene (RI 1487, Rt: 9.18) (Figure 3). Several
94 peaks with a *m/z* of 220 were also observed but not identified. A hexane extract of *N. benthamiana* leaves,
95 expressing *TITPS509* was analyzed by GC-MS using a Programmed Temperature Vaporising injector (PTV)
96 port. By using a low initial temperature of 60°C followed by a gradual increase to 250°C it was expected that
97 thermal rearrangements were avoided. Again, **3** and **4** were detected as major compounds, but not guaiene and
98 bulnesene, which were probably below the detection limit. Furthermore, neither of the unknown compounds
99 with *m/z* 220 were detected in the PTV analysis.

2.3.1. Isolation and structure elucidation of the *TITPS509* products, bulnesol and guaiol

In order to verify the identity of **3** and **4** by NMR, the compounds were isolated in two purification steps. An initial purification of the compounds was performed on a silica column. **3** and **4** both eluted with 10% ethyl acetate in hexane and this fraction was further fractionated on a preparative GC-MS and **3** and **4** were separated and both precipitated as a colorless oil in the fraction collector. The ¹H and ¹³C NMR resonance values of **3** and **4** were all in accordance with those reported by Raharivelomanana et al., (1995), which confirm the identity of **3** and **4**. The spectra are provided in Supplementary data figure 12 and 13.

2.4. Characterization of *TITPS18983* expressed in *N. benthamiana*

Volatiles from *N. benthamiana* leaves expressing *TITPS18983* were captured by HS-SPME and analyzed by GC-MS. The analysis of these plants showed that expression of *TITPS18983* generate at least 15 products. The identification of one of the major products, farnesene (**5**), was based on comparison of the mass spectrum of the compound with NIST and Wiley GC-MS spectrum libraries (Figure 4, MS spectrum is provided in supplementary data Figure 3). It was not possible to identify the remaining products. Using an injection port temperature of 160 °C instead of 250 °C did not alter the composition of the chromatogram, which supports that these compounds are not a result of the GC-MS analysis method. Extraction of the products was also attempted using organic solvents such as hexane and pentane mimicking procedures that were successful for other sqTPS products. However, for *TITPS18983* we did not detect any compounds by GC-MS using organic solvent extraction.

2.5. Characterization of *TICYP76AE4* using epikunzeol from *TITPS820* as substrate

To investigate if *TICYP76AE4* would be able to use **1** as a substrate, *TITPS820* and *TICYP76AE4* were co-expressed in *N. benthamiana*. This resulted in complete conversion of **1** as seen in Figure 1. One new product was detected in the GC-MS chromatogram with an injection port temperature of 250°C. However, the peak appeared to consist of two compounds that were co-eluting on the GC-MS. It was not possible to separate these products further. The GC-MS analysis indicated that the parent ions had a *m/z* of 238. To further support that *TITPS820* yields **1**, *TICYP76AE4* was also expressed with *TgTPS2* and was shown to yield the same new product(s) (supplementary data Figure 1). From the chromatograms and spectrums, it was clear that *TICYP76AE4* yielded a new product. Co-expression of *TgTPS2*, *TgCYP76AE2* and *TICYP76AE4* did not result in additional new products.

2.5.1. Isolation and structure elucidation of tovarol

Compound **2** was isolated from the hexane extract of leaves of *N. benthamiana* expressing *TITPS820* and *TICYP76AE4*. The isolation was performed by semi-preparative normal-phase HPLC and the purity of **2** was confirmed by LC-HRMS. High resolution mass spectrometry (HRMS) established the accurate mass of **2** to *m/z* 261.1826 ([M+Na]⁺) to which the molecular formula was deduced to be C₁₅H₂₆O₂ (calcd. for C₁₅H₂₆O₂

133 [M+Na]⁺ *m/z* 261.1825, Δ -0.2 ppm). **2** was structurally elucidated by interpretation of the ¹H, ¹³C and ¹H-¹H
134 COSY, ¹H-¹³C ed-HSQC, ¹H-¹³C HMBC and ¹H-¹H ROESY NMR data. The data for **2** were in agreement
135 with the germacradiendiol tovarol (Figure 2), which has previously been isolated from *Thapsia* species (De
136 Pascual Teresa et al., 1985; Teresa et al., 1986). Due to the low quality of the published spectra and the absence
137 of ¹³C NMR data a full structure elucidation was performed. The DEPT spectrum of the metabolite revealed
138 that **2** contained two quaternary carbon atoms, six methine carbon atoms, three methylene groups and four
139 methyl groups. Even though most of the peaks in the recorded spectrum were broad, presumably because of a
140 conformational equilibrium (Fischer et al., 1979; Triana et al., 2005), an in-depth investigation was possible
141 (Table 1). The use of 2D spectra also enabled an assignment of the signals in the ¹³C NMR spectrum. The *E,E*-
142 configuration was established through a ROESY correlation between H-1 and H-5. Model building reveals
143 that only the *E,E*-configuration allows H-1 and H-5 to be near in space. ROESY correlation between H-6, H-
144 15 and H-7 revealed that H-6 and H-7 were *cis*-disposed. Comparison with a reference spectrum of tovarol
145 (Prof. G. Appendino personal communication) revealed that previous isolated tovarol possessed the same
146 stereochemistry at C-8 as **2**, and revealed that the hydroxyl group at C-8 is α-disposed. A ROEYS correlation
147 between H-7 (1.05 ppm) and H-9 resonating at 1.78 ppm revealed that this H-9 must be α-disposed and
148 consequently the signal at 2.49 ppm was H-9β. The absolute configuration was assumed based on the
149 observation that sesquiterpenes from Apiaceae plants have H-7 α-disposed (Fischer et al., 1979; Simonsen et
150 al., 2013). **2** is therefore concluded to be tovarol (De Pascual Teresa et al., 1985).

151 **3. Discussion**

152 A variety of sesquiterpenoids are produced in *Thapsia* and the sesquiterpenoid profile often differs between
153 species (Christensen et al., 1997). Previous analysis of *T. garganica* and *T. laciniata* have shown that these
154 species produce sesquiterpenoids with different backbones and side-group modifications (Drew et al., 2012).
155 *T. laciniata* has at least four functional sesquiterpene synthases and two of them, *TTPS18983* and *TTPS509*,
156 are multi-product synthases. Compared to the two sqTPS found in *T. garganica* this correlates well with the
157 higher number of metabolites described in *T. laciniata* (Christensen et al., 1997; Drew et al., 2012, 2013). In
158 *Daucus carota* 20 sqTPS (TPS-a family) are predicted, which show that even between closely related species
159 the number of sqTPS's can vary tremendously (Banasiak et al., 2016; Keilwagen et al., 2017).

160 Several farnesene synthases have been described in literature. *T118983* is however the first farnesene synthase
161 from Apiaceae. The finding of a farnesene synthase is supported by the previous detection of β-farnesene in
162 *T. laciniata* (Drew et al., 2012). It is interesting that *T118983* is able to produce a large number of products,
163 which cannot merely be characterized as minor side products, at least judging by expression in *N. benthamiana*.
164 Analyzing a multiproduct sqTPS is complex due to the difficulty of separating the products by GC-MS, and
165 due to very volatile nature of these compounds. Sesquiterpenoids in many species are a part of the volatile
166 mixture of compounds that is emitted by the plants. It can be hypothesized that the products produced by

167 *TTPS18983* are a part of the volatile signaling compounds of *T. laciniata*, as also seen in other plants (Flamini
168 et al., 2003; Máday et al., 1999; Wang et al., 2015).

169 Two major products of *TTPS509* were identified as guaiol (**3**) and bulnesol (**4**). Previous investigation of *T.*
170 *laciniata* sesquiterpenoids (Drew et al., 2012; Lemmich et al., 1984) also showed that **3** is produced by this
171 species. Three guaiene esters, (4*S*, 5*S*, 7*S*, 8*S*)-8-*p*-Coumaroyloxy-1(10)-guaiene-11-ol, (4*S*, 5*S*, 7*S*, 8*S*)-8-
172 Feruloyloxy-1(10)-guaiene-11-ol, and (4*S*, 5*S*, 7*S*, 8*S*)-8-Seneciolyoxy-1(10)-guaiene-11-ol have also been
173 found in *T. laciniata* (Lemmich et al., 1991). All of these are derived from the structure of **4** and have different
174 side chain modifications at C-8. This indicates that bulnesol is a possible precursor of these three structures.
175 The described modifications at C-8 are expected to involve a hydroxylation at C-8 followed by the addition of
176 side chains by acyl transferases. Currently, *T. laciniata* is the only species in the genus *Thapsia* that has been
177 found to produce guaiols (Christensen et al., 1997). Furthermore, α -guaiene was previously found in *T.*
178 *laciniata* (Drew et al., 2012). This is a minor product of *TTPS509* and is the non-hydroxylated compound
179 with the same type of 5- and 7-ring structure as guaiols. The same is also observed for bulnesene and bulnesol.
180 Co-expression of CYP76AE4 and *TTPS509* did not provide any new products. Thus, an orthologue of
181 CYP76AE4 might be responsible for the hydroxylation at C-8, though this remains to be examined.

182 *TTPS820* produces epikunzeaol (**1**) as a main product. The discovery of the two orthologues sqTPS genes
183 (*TgTPS2* and *TTPS820*) in *T. laciniata* and *T. garganica* shows that the epikunzeaol synthase can serve as a
184 marker for the *Thapsia* genus. It also shows that the species within *Thapsia* have evolved different downstream
185 products by using **1** as a substrate. While *TgCYP76AE2* and *TICYP76AE4* are both able to use **1** as a substrate,
186 they perform hydroxylation on different carbon positions. This difference is also reflected in the metabolite
187 profiles of the two species. In contrast to most *Thapsia* species, *T. laciniata* does not contain guaianolides, for
188 which epidihydrocostunolide (the product of *TgCYP76AE2* in *T. garganica*) appears to be a precursor
189 (Andersen et al., 2017). Tovarol has currently not been detected from *T. laciniata* and could be involved in the
190 biosynthesis of a downstream product yet to be discovered. However, tovarol and several derivatives was
191 isolated from the closely related *Thapsia minor* Hoffmanns. & Link (De Pascual Teresa et al., 1985). It is likely
192 that similar biosynthetic routes are present in *T. laciniata* and *T. minor* since these two species are very closely
193 related (Banasiak et al., 2016; Weitzel et al., 2014). It remains to be investigated if *T. minor* harbors orthologue
194 genes to those described here and in *T. garganica*.

195 The products detected by GC-MS from *TgCYP76AE2* were shown to be Cope re-arrangements of
196 dihydrocostunolide (Andersen et al., 2017). It is highly probable that **2** from *TICYP76AE4* has also undergone
197 Cope re-arrangement during exposure to the high injection port temperature in the GC-MS (Figure 1). Through
198 the isolation of **2** it was clear from our LC data that *TICYP76AE4* only produces **2** as the product.

199 The expression of genes, involved in sesquiterpene biosynthesis in *N. benthamiana* has previously been
200 reported to result in the conjugation of produced sesquiterpenoids. This was shown for artemisinic acid in the
201 artemisinin pathway, which was conjugated to a diglucoside (van Herpen et al., 2010). In costunolide
202 biosynthesis, the analysis was affected by the conjugation of costunolide to glutathione or cysteine (Liu et al.,
203 2011). LC-MS analysis of **2** from *TlCYP76AE4* did not reveal any conjugation to the two alcohol groups,
204 which therefore allowed for detection by GC-MS as well. **1** on the other hand from *TlTPS820* and *TgTPS2*
205 was found both in its free form in GC-MS analysis and as a disaccharide in LC-MS analysis.

206

207 **4. Conclusion**

208 We have described the activity of three sesquiterpene synthases from *Thapsia laciniata*. *TlTPS820* was found
209 to be an epikunzeaol synthase whereas *TlTPS18983* and *TlTPS509* are multi-product sesquiterpene synthases,
210 with farnesene as the major product of *TlTPS18983* and guaïol and bulnesol as the major products of
211 *TlTPS509*. *TlTPS820* is an orthologue to *TgTPS2* from *Thapsia garganica* and co-expression with the
212 cytochrome P450 *TlCYP76AE4* led to the biosynthesis of tovarol. Thus, we have shown that *TlCYP76AE4*
213 hydroxylate epikunzeaol at C-8 to yield tovarol.

214

215 **5. Experimental section**

216 **5.1. Plant material**

217 *Thapsia laciniata* Rouy, Apiaceae (Banasiak et al., 2016; Weitzel et al., 2014) roots were collected in early
218 June 2008, just west of Cannes, France (GPS 43.540958, 6.816158). Voucher specimens of *T. laciniata* (HTS
219 2008-01) are stored at herbarium C (Natural History Museum of Denmark, Copenhagen).

220 **5.2. Identification and cloning of genes**

221 The sesquiterpene synthases *TlTPS18983* (MG680745), *TlTPS509* (MG680746) and *TlTPS820* (MG680744)
222 were found in a transcriptome dataset from *T. laciniata* root (SRX252523) (Drew et al., 2013). The full-length
223 sequences were obtained from a cDNA library from *T. laciniata* root material. The discovery of *TgTPS2*
224 (JQ290345) and *TgCYP76AE2* (KX826943) has been presented previously (Andersen et al., 2017; Pickel et
225 al., 2012). *TgCYP76AE2* was blasted into the transcriptome dataset to search for a homologues sequence. The
226 resulting P450 was named, *TlCYP76AE4* (MG680747) (Nelson, 2009).

227 Forward and reverse primers for all genes were designed with USER-overhangs, to enable cloning into a
228 pEAQ-USER compatible version of the pEAQ-*HT* vector (Supplementary data, table 1) (Luo et al., 2016).

229 pEAQ-*HT* harbors the viral suppressor p19 and was kindly provided by George Lomonosoff (John Innes
230 Research Centre, Norwich, UK) (Peyret and Lomonosoff, 2013). USER cloning was performed as previously
231 depicted (Nour-Eldin et al., 2006).

232 **5.3. Expression of *TITPS18983*, *TITPS509*, *TgTPS2*, *TITPS802*, *TgCYP76AE2* and** 233 ***TICYP76AE4* in *Nicotiana benthamiana***

234 *Nicotiana benthamiana* plants were grown from seeds at 24 °C / 19 °C (day/night) for 5 weeks before
235 transformation. The transformation of *Agrobacterium tumefaciens* and infiltration of *N. benthamiana* with *A.*
236 *tumefaciens* was achieved as previously described (Bach et al., 2014). Plants were harvested 5 days after
237 infiltration. In short, 10 ml LB containing kanamycin, rifampicin and carbenicillin was inoculated with several
238 agrobacterium colonies containing the plasmid of interest. Cultures were grown overnight at 28 °C and 200
239 rpm. Cell pellets were washed twice with water before a final resuspension in water followed by a dilution to
240 OD₆₀₀ 0.5. Resuspended *A. tumefaciens* carrying plasmids containing *AtHMGR*, *TITPS* or *TICYP76AE4* were
241 mixed 1:1:1 and infiltrated into leaves of at least three *N. benthamiana* plants by use of a syringe. Plants were
242 placed at 24 °C / 19 °C (day/night) and harvested five days after infiltration. As controls, plants were infiltrated
243 with *A. tumefaciens* carrying plasmids with *AtHMG*, or *AtHMGR* plus *TITPS*s.

244 The ~100 plants needed for purification of tovarol were infiltrated by use of vacuum. Three *A. tumefaciens*
245 cultures containing *AtHMGR*, *TITPS820* or *TICYP76AE4* were grown overnight at 28 °C and 200 rpm in 500
246 ml LB (containing kanamycin, rifampicin and carbenicillin) from 20 ml starter cultures. Cell pellets were
247 washed twice with water before final resuspension in water followed by a dilution to OD₆₀₀ 0.5. Resuspended
248 *A. tumefaciens* carrying plasmids containing *AtHMGR*, *TITPS820* or *TICYP76AE4* were mixed 1:1:1. Plants
249 were submerged in a 1 L suspension of *A. tumefaciens* and infiltrated by use of vacuum at 50-100 mbar for 1
250 min (Andersen-Ranberg et al., 2016).

251 **5.4. GC-MS detection of sesquiterpenoids from liquid extractions**

252 Two leaf discs (diameter 3 cm) from *N. benthamiana*, expressing genes from *T. laciniata* were extracted with
253 1.2 ml hexane for GC-MS analysis to provide one sample, a minimum of three biological replicates were
254 examined. Samples were analyzed on a Shimadzu GCMS-QP2010. The column used was an Agilent HP-5MS
255 UI, 20 m, 0.18 mm diameter × 0.18 µm film thickness. The pressure was kept at 66.7 kPa giving a column
256 flow of 1 mL/min. The injection port temperature was set to 250 °C. The oven temperature was set to 60 °C
257 for 3 min, and then increased to 160 °C with a rate of 7 °C/min. The temperature was further increased to 300
258 °C at a rate of 50 °C /min, held for 5 min, finally increased to 320 °C at 50 °C/min and maintained for 3 min.
259 The carrier gas was H₂ and the ionization electron energy was 70 eV. The ion source temperature was 230 °C
260 with an interface temperature 280 °C. The total run time was 28.49 min. All data were analyzed using the

261 Shimadzu software Lab Solutions, GCMS Solutions version 2.50 SU3, with the 2008 libraries provided by
262 NIST and Wiley.

263 **5.5. HS-SPME-GC-MS analysis of sesquiterpenoids**

264 *TTPS18983* and *TTPS509* were also analyzed on GC-MS by use of HS-SPME fibers. HS-SPME fibers were
265 purchased from Supelco Sigma-Aldrich, Denmark. A whole leaf was placed in a 20 ml glass vial and extracted
266 at 60 °C for 20 min with a SPME fiber exposed to the air above the leaf (Andersen et al., 2015a). Immediately
267 hereafter, the SPME fiber was run on the GC-MS. Samples were analyzed on a Shimadzu GCMS-QP2010.
268 The column used was an Agilent HP-5MS UI, 20 m, 0.18 mm diameter × 0.18 µm film thickness. The pressure
269 was kept at 66.7 kPa giving a column flow of 1 mL/min. The injection port temperature was set to 160 °C or
270 250 °C. The oven temperature was set to 60 °C for 3 min, and then increased to 160 °C with a rate of 7 °C/min.
271 The temperature was further increased to 300 °C at a rate of 50 °C/min, held for 5 min, finally increased to
272 320 °C at 50 °C/min and maintained for 3 min. The carrier gas was H₂ and the ionization electron energy was
273 70 eV. The ion source temperature was 230 °C with an interface temperature 280 °C. The total run time was
274 28.49 min. All data were analyzed using the Shimadzu software Lab Solutions, GCMS Solutions version 2.50
275 SU3, with the 2008 libraries provided by NIST and Wiley.

276 **5.6. Analytical LC-MS detection of sesquiterpenoids**

277 Two leaf discs (diameter 3 cm) were ground in liquid nitrogen. Samples were extracted with 1200 µl 80 %
278 methanol and sonicated for 30 min. Before LC-MS analysis samples were filtered through a 0.45 µm filter.
279 Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Santa Clara, CA,
280 USA) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany).
281 Separation was achieved on a Gemini-NX C₁₈ column (Phenomenex; 2×150 mm, 3 µm) maintained at 35 °C.
282 The mobile phases were: A, water with 0.1 % (v/v) HCOOH; B, acetonitrile with 0.1 % (v/v) HCOOH. The
283 gradient program was: 0 to 1 min, isocratic 12 % B; 1 to 33 min, linear gradient 12 to 80 % B; 33 to 35 min,
284 linear gradient 80 to 99 % B; 35 to 38 min, isocratic 99 % B; 38-47 min, isocratic 12 % B. The flow rate was
285 0.2 mL/min. The mass spectrometer was operated in positive mode and the range *m/z* 100-1000 was acquired.

286 High-resolution mass spectrometry of **2** was acquired on a maXis HD mass spectrometer (Bruker Daltonics,
287 Bremen, Germany) coupled to an Ultima 3000 series HPLC-DAD (Thermo, Waltham, Massachusetts, USA).
288 Separation was achieved on a Kinetex C₁₈ column (Phenomenex, Torrance, USA, 150×2 mm, 2.6 µm) using
289 a linear gradient consisting of A, H₂O with 20 mM HCOOH and B, HPLC-MS grade MeCN with 20 mM
290 HCOOH, from 10-100 % B in 10 min with a flow rate of 0.4 ml/min, the column was maintained at 40 °C.
291 The mass spectrometer was operated in positive ESI acquiring the *m/z* range of 75-1250.

292 **5.7. Isolation of guaiol and bulnesol from *TTPS509* by preparative GC-MS**

293 For isolation of guaiol and bulnesol, a large-scale hexane extraction was made from *N. benthamiana* leaves
294 expressing truncated *AtHMGR* and *TTPS509*. The leaves from approximately 100 five-week-old plants were
295 extracted with hexane. The crude hexane extract was subjected to an initial preparative separation on a silica
296 column and eluted stepwise in hexane-ethyl acetate, from hexane to hexane:ethyl acetate (80:20) with 2 %
297 increments. The final purification of products from *TTPS509* were performed on a preparative GC-MS using
298 an Agilent 7890B GC installed with an Agilent 5977A inert MSD, GERSTEL Preparative Fraction Collector
299 (PFC) AT 6890/7890 and a GERSTEL CIS 4C Bundle injection port. For separation by GC, a RESTEK Rtx-
300 5 column (30m x 0.53mm ID x 1 μ m df) with H₂ as the carrier gas was used. A split piece with a split of 1:100
301 (MS:PFC) was placed at the end of this column. Sufficient amounts of sesquiterpene product for NMR analysis
302 (0.5-1 mg) were obtained with 5 μ L injections of extract repeated 100 times. The injection port was put in
303 solvent vent mode with a carrier gas flow of 100 mL/min until minute 0.17, combined with an injection speed
304 of 1.5 mL/min. The purge flow was set to 3 mL/min from minute 0.17 to 2.17. The injection temperature was
305 held at 40 °C for 0.1 min followed by ramping at 12 °C/sec until 320 °C, which was then held for 2 min. The
306 column flow was set to 7.5 mL, which was held constant throughout the GC program. The GC program was
307 set to hold at 60 °C for 1 min, ramp 20 °C/min to 320 °C and 320 °C held for 3 min. The temperature of the
308 transfer line from GC to PFC and the PFC itself was set to 250 °C. The PFC was set to collect the selected
309 peaks based on the MS spectrum. The MS was set in scan mode from *m/z* 35 to *m/z* 500, with a threshold of
310 150. Solvent cut-off was set to 4 min, and the temperature of the MS source and the MS quadrupole was set to
311 300 °C and 150 °C, respectively. Traps were kept at -20°C, in case products were prone to rearrangement, and
312 rinsed with chloroform-d (Euriso-top, 99.8 atom % D).

313 **5.8. Isolation of tovarol from *TICYP76AE4* by HPLC**

314 For isolation of tovarol, a large-scale hexane extraction was made from *N. benthamiana* leaves expressing
315 truncated *AtHMGR*, *TTPS820* and *TICYP76AE4*. The leaves from approximately 100 five-week-old plants
316 were extracted with hexane. The crude hexane extract was subjected to an initial preparative separation on a
317 Isolera autoflasher using a 10 g diol functionalized silica column (Biotage, Uppsala, Sweden) eluted stepwise
318 in hexane-ethyl acetate, from hexane to hexane:ethyl acetate (80:20) with 2 % increments. Final isolation of
319 tovarol was achieved by semi-preparative HPLC utilizing a 250 × 10 mm, 7 μ m Nucleosil PEI column
320 (Macherey-Nagel) eluted in a linear hexane-isopropanol gradient from 0-5 % isopropanol in 20 min, on a
321 Waters 600 HPLC equipped with a Waters 996 PDA detector. The purity was confirmed by LC-MS as
322 described in 5.6.

5.9. ¹H and ¹³C NMR spectroscopic analysis

NMR-spectra were acquired using a 600 MHz Bruker Avance III HD NMR spectrometer (¹H operating frequency 600.13 MHz) equipped with a Bruker SampleJet sample changer and a cryogenically cooled gradient inverse triple-resonance 1.7-mm TCI probe-head (Bruker Biospin, Rheinstetten, Germany) optimized for ¹³C and ¹H. Samples were analyzed at 300 K. Proton spectra, at 600.03 MHz, were obtained using 30°-pulses, a spectral width of 12 kHz, collecting 16 scans with a length of 65536 data points with a relaxation delay of 1.0 sec. Carbon spectra were acquired at 150.88 MHz with 30°-pulses, a spectral width of 36 kHz, collecting 256 scans with a length of 65536 data points and with a relaxation delay of 2.0 sec. The ¹³C nuclei were ¹H-decoupled using the Waltz-16 composite pulse-decoupling scheme. FID's were exponentially multiplied with a line-broadening factor of 1.0 Hz before Fourier transformation. The 2D experiments were recorded using Bruker standard parameter settings. The isolated tovarol was dissolved in MeCN-d₃ (99.8 atom % D) prior to NMR analysis.

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345 **Figure legends**

346 **Figure 1:** GC-MS chromatogram of hexane extracts from *N. benthamiana* expressing *AtHMGR*+ *TTPS820*,
347 *AtHMGR*+*TgTPS2*, and *AtHMGR*+ *TTPS820* + *TICYP76AE4*. The chromatograms show that *TgTPS2* and
348 *TTPS820* both produce epikunzeaol (**1**) as previously published for *TgTPS2* (Andersen et al., 2017), and with
349 co-expression of *TICYP76AE4* this product disappear and a cope rearranged product of tovarol (**2**) is found.
350 This confirms the functionality of both enzymes.

351 **Figure 2:** The biosynthetic routes catalyzed by the four described *Thapsia laciniata* enzymes *TTPS820*,
352 *TTPS509*, *TTPS18983*, and *TICYP76AE4* leading to tovarol (**2**).

353 **Figure 3:** HS-SPME GC-MS analysis of *TTPS509* expressed together *AtHMGR*, the control shows the
354 expression of *AtHMGR* alone. Guaiol (**3**) is seen as the major product (Rt 9.89 min), with bulnesol (**4**), Rt:
355 10.2, a-guaiene (Rt: 8.6), and a-bulnesene (Rt: 9.18) as minor products. * show unidentified sesquiterpene like
356 products, which is either ketone formed during the biosynthesis or breakdown products of the alcohols formed
357 during the GC-MS analysis. Based on this *TTPS509* is a guaiol synthase. MS spectra are presented in
358 supplementary data

359 **Figure 4: a)** HS-SPME GC-MS analysis of *TTPS18983* expressed together *AtHMGR*, the control sample
360 show the expression of *AtHMGR* alone. farnesene (**5**) is seen as the major product (Rt 8.75 min), * show
361 unidentified sesquiterpene like products. Based on this *TTPS18983* is a multiproduct terpene synthase with
362 farnesene as the major product. MS spectra are presented in supplementary data.

363

364 **Tables**

365 Table 1: NMR data for tovarol (**2**). The spectrum was recorded in CD₃CN at 600 MHz. Most of the signals are
 366 broad, which is probably because of a conformational equilibrium. Numbering is shown in Figure 2.

Position	δ_{H} mult (<i>J</i> Hz)	δ_{C} type
1	4.96, br.qd (<i>J</i> 12.0, 5.1)	131.3 CH
2 β	2.39, dd, (<i>J</i> 12.0, 5.1)	25.6 CH ₂
2 α	2.06, ddt, (<i>J</i> 12.0, 5.1, 2.0)	
3	2.14, br.dd, (<i>J</i> 12.0, 5.1)	39.7 CH ₂
	2.06, br.ddt, (<i>J</i> 12.0, 5.1, 2.0)	
4		132.3 C
5	5.13, br.d (<i>J</i> 6.6)	133.6 CH
6	4.57, br.d (<i>J</i> 6.6)	69.2 CH
7	1.05, overlaid	54.7 CH
8	4.16, br.ddd, (<i>J</i> 11.7, 5.9, 0.7)	73.2 CH
9 β	2.49, dd (<i>J</i> 11.9, 6.0)	45.6 CH ₂
9 α	1.78, t (<i>J</i> 11.9)	
10		135.5 C
11	2.00 dq, (<i>J</i> 10.3, 6.6)	26.4 CH
12	1.02, d, (<i>J</i> 6.6)	21.9 CH ₃
13	1.05, d, (<i>J</i> 6.2) overlaid	21.4 CH ₃
14	1.64, s	24.2 CH ₃
15	1.42, s	16.8 CH ₃
OH	2.14, br.s overlaid	

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369 **7. References**

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