Biosynthesis of Diterpenoids in *Tripterygium* Adventitious Root Cultures

Fainmarinat S. Inabuy, Justin T. Fishedick, Iris Lange, Michael Hartmann, Narayanan Srividya, Amber N. Parrish, Meimei Xu, Reuben J. Peters, and B. Markus Lange*

Institute of Biological Chemistry and M.J. Murdock Metabolomics Laboratory, Washington State University, Pullman, WA 99164-6340, USA (A.M.P., B.M.L., F.S.I., J.T.F., M.H., N.S.); Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011-1079, USA (M.X., R.J.P.)

This work was supported by the National Institutes of Health (award numbers RC2GM092561 to B.M.L. and GM076324 to R.J.P.) and McIntire-Stennis formula funds from the Agricultural Research Center at Washington State University (to B.M.L.).

Present address: Excelsior Analytical, Union City, CA 94587, USA.

Present address: Institute of Molecular Ecophysiology of Plants, Heinrich Heine Universität Düsseldorf, 40225 Düsseldorf, Germany.

*Correspondence* (Tel +1 509 335 3794; fax +1 509 335 3794; email lange-m@wsu.edu)
ONE SENTENCE SUMMARY
Adventitious root cultures provide insights to elucidating the biosynthesis of pharmaceutically relevant diterpenoids in the model genus Tripterygium

AUTHOR CONTRIBUTIONS
B.M.L. conceived of the project. F.S.I. cloned candidate genes, subcloned them into appropriate modular expression vectors and analyzed diterpenoids produced in E. coli cells harboring these constructs. J.T.F. established the methods for the isolation, purification, identification and quantitation of diterpenoids. I.L. isolated RNA from adventitious root cultures and cloned candidate genes. In addition, I.L. performed the scale-up and selection of adventitious root cultures for high diterpenoid production. M.H. established and A.N.P. maintained the adventitious root cultures. N.S. performed the functional characterization of monoterpene synthase genes. M.X. and R.J.P. supplied modular vectors and carried out diterpenoid characterizations. B.M.L. and F.S.I. wrote the manuscript, with contributions from all authors.

Key words: diterpene synthase, diterpenoid, monoterpene synthase, tissue culture, triterpenoid

Abbreviations and Acronyms: CPP, copalyl diphosphate; CPS, copalyl diphosphate synthase; eKS, ent-kaurene synthase; GGPPS, geranylgeranyl diphosphate synthase; HPLC-QTOF-MS, high performance liquid chromatography-quadrupole time of flight-mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; TPS, terpene synthase.
ABSTRACT

Adventitious root cultures were developed from *Tripterygium regelii* Sprague & Takeda and growth conditions optimized for the abundant production of diterpenoids, which can be collected directly from the medium. An analysis of publicly available transcriptome data sets collected with *T. regelii* roots and root cultures indicated the presence of a large gene family (with 20 members) for terpene synthases (TPSs). Nine candidate diterpene synthase genes were selected for follow-up functional evaluation, of which two belonged to the TPS-c, three to the TPS-e/f and four to the TPS-b subfamily. These genes were characterized by heterologous expression in a modular metabolic engineering system in *E. coli*. Members of the TPS-c subfamily were characterized as copalyl diphosphate (diterpene) synthases and those belonging to the TPS-e/f family catalyzed the formation of precursors of kaurane diterpenoids. The TPS-b subfamily encompassed genes coding for enzymes involved in abietane diterpenoid biosynthesis and others with activities as monoterpene synthases. The structural characterization of diterpenoids accumulating in the medium of *T. regelii* adventitious root cultures, facilitated by searching the Spektraris online spectral database, enabled us to formulate a biosynthetic pathway for the biosynthesis of triptolide, a diterpenoid with pharmaceutical potential. Considering the significant enrichment of diterpenoids in the culture medium, fast-growing adventitious root cultures may hold promise as a sustainable resource for the large-scale production of triptolide.
INTRODUCTION

Tripterygium wilfordii Hook. f., also known as léi gōng téng in Mandarin Chinese (generally translated as “thunder god vine”), has a long history of use in traditional Chinese Medicine for the treatment of fever, chills, edema, and carbuncles (Helmstädter, 2013). The genus Tripterygium (Celastraceae) is known to be a rich source of specialized metabolites, of which more than 400 have been isolated, structurally characterized, and assessed in cell-based assays (Brinker et al., 2007). Root extracts have been evaluated as a medication for rheumatoid arthritis, cancer, hepatitis, nephritis, ankylosing spondylitis, polycystic kidney disease, and obesity; more than a dozen clinical trials with such extracts (often referred to as “Tripterygium glycoside”) have been completed but, in part due to shortcomings in study designs, the efficacy has remained a matter of debate (Chen et al., 2010; Liu et al., 2013; Zhu et al., 2013). More promising results have been obtained with purified constituents, which are usually extracted from Tripterygium roots. Semi-synthetic chemical derivatives of triptolide, a diterpenoid epoxide, have been evaluated in phase I clinical trials (Meng et al., 2014; Zhou et al., 2012). Minnelide, a water soluble pro-drug analogue of triptolide, has shown very promising activity in multiple animal models of pancreatic cancer (Chugh et al., 2012) and, in 2013, was advanced to phase I clinical trials in the United States (clinicaltrials.gov identifier NCT01927965).

One of the critical challenges for clinical evaluations is a supply shortage for triptolide and other diterpenoids. The most abundant specialized metabolites in Tripterygium roots are quinone methide triterpenoids and sesquiterpene pyridine alkaloid macrolides, whereas triptolide and other diterpenoids occur only at very low concentrations ranging from 0.0001 to 0.002 % of dry weight biomass (Guo et al., 2014; Zeng et al., 2013; Zhou et al., 2012). The extraction yields of diterpenoids from Tripterygium roots are accordingly poor and alternative, sustainable production methods need to be developed. Tissue cultures represent a promising alternative for the production of high value plant metabolites. Early studies with T. wilfordii suspension cultures were designed to unravel the structures of small molecule constituents (Kutney and Han, 1996; Kutney et al., 1981; Kutney et al., 1992; Kutney et al., 1993; Nakano et
Similarly, hairy root cultures were initially employed for phytochemical investigations (Nakano et al., 1998). It was recognized only recently that triptolide concentrations produced by tissue cultures (up to 0.15 % of dry weight) (Miao et al., 2013; Miao et al., 2014; Zhu et al., 2014) far exceed those reported for roots. 

Surprisingly, despite considerable pharmaceutical interest, the biosynthesis of triptolide has only recently attracted the deserved attention. Several diterpene synthases, some of which are relevant to triptolide formation, have now been characterized in *T. wilfordii* (Andersen-Ranberg et al., 2016; Hansen et al., 2017; Zerbe et al., 2013). However, the remaining genes involved in the biosynthesis of the highly functionalized triptolide structure have remained enigmatic. In this manuscript, we describe the development of *Tripterygium* adventitious roots cultures in which diterpenoids are produced as principal metabolites that can be harvested sustainably from the medium. Based on the structures of these metabolites, we can now postulate the biochemical steps leading to the main diterpenoid metabolites. In addition, analysis of transcriptome data sets, acquired with diterpenoid-producing adventitious root cultures, enabled the selection and subsequent functional characterization of genes involved in the early steps of diterpenoid biosynthesis. Our results indicate that *Tripterygium* tissue cultures offer opportunities to both unravel diterpenoid biosynthetic pathways and produce target diterpenoids – such as triptolide – at larger scale.

**RESULTS**

**Development of Diterpenoid-Secreting Adventitious Root Cultures**

*Tripterygium regelii* Sprague & Takeda adventitious roots cultures were initiated based on standard protocols. Over a one-year period, during which medium was extracted with ethyl acetate and hydrophobic metabolites were analyzed by High Performance Liquid Chromatography – Quadrupole Time-Of-Flight – Mass Spectrometry (HPLC-QTOF-MS), the highest triptolide producers were selected for further propagation. In contrast to roots of mature *Tripterygium* plants, where triterpenoids occur at low concentrations (< 1 %) and diterpenoids are trace constituents (< 0.01 %), our
adventitious root cultures accumulated diterpenoids abundantly in the medium and triterpenoids (in particular celastrol) in roots (Fig. 1A, B). By comparing adjusted peak areas from HPLC-QTOF-MS runs, we estimated that diterpenoids constitute approximately 77% of all detected metabolites in an organic extract of the culture medium (Supplemental Fig. S1).

An accurate quantitation of all diterpenoids was not possible because we did not have a complete set of authentic standards, but we performed absolute quantitations for signature metabolites (triptolide and celastrol). Various common reagents and elicitors were tested (methyl jasmonate; methyl salicylate, chitosan, yeast extract, and cold exposure), but only the methyl salicylate treatment resulted in a significant increase in triptolide accumulation (2.2-fold; $P$-value 0.01) and concomitant decrease in celastrol concentration (Supplemental Table S1). The disadvantage of methyl salicylate treatments was that root color darkened and growth ceased, often for months. We therefore focused our efforts on further selecting cultures that were high diterpenoid producers under non-inducing conditions, and could be maintained for extended periods of time. These cultures were gradually scaled up to 2.8 L flasks (500 mL medium) (Fig. 1C). Triptolide accumulated at 4.7 mg L$^{-1}$ (Fig. 1D), and was readily extractable from the culture medium every two weeks (when adventitious roots were transferred to fresh media).

Hundreds of structurally diverse metabolites have been isolated and characterized from various *Tripterygium* organs and tissues (Lange et al., 2017). Our adventitious root cultures, in contrast, secreted primarily diterpenoids into the culture medium and are therefore an excellent experimental model system to study the biosynthesis of these clinically relevant metabolites. As a first step to further assess the potential of these tissue cultures, it was important to identify the major constituents of medium extracts. Following HPLC fractionation of medium extracts of *Tripterygium* adventitious root cultures, fractions containing metabolites that corresponded to prominent peaks in HPLC-QTOF-MS chromatograms were characterized by MS, MS/MS and $^1$H-NMR spectroscopy (Supplemental Fig. S1 and Supplemental Methods and Data File S1), and metabolites identified by searches against the Spektraris database (Fischiedick et al., 2015). We ascertained the identify of five diterpenoids, were able to annotate three
additional metabolites with high confidence, and acquired tentative identifications for an additional two metabolites (Table 1). Three metabolites showed the mass fragmentation patterns typical of diterpenoids but their identity remained unknown (Table 1).

**Functional Characterization of Candidate Diterpene Synthases**

For obtaining full-length candidate genes, we deemed it advantageous to employ all available *T. regelii* sequences. We therefore downloaded several publicly available transcriptome data sets obtained with roots and root cultures, and then generated a consensus assembly. tBLASTn searches with peptide sequences of diterpene synthases of dicotyledons were performed against our assembly data. A phylogenetic analysis indicated that translated peptide sequences of two *T. regelii* genes (TrTPS2 and TrTPS1) clustered with functionally characterized class II diterpene synthases of the TPS-c family (copalyl diphosphate synthases) (Fig. 3 and Supplemental Table S2). Full-length cDNAs were cloned (KX533964 and KX533965, respectively) (see Supplemental Table S3 for primer sequences) and their biochemical function evaluated (see below). Translated peptide sequences of three genes (TrTPS13, TrTPS14 and TrTPS15) clustered with functionally characterized class I *ent*-kaurene synthases of the TPS-e/f family (Fig. 3). Full-length clones (KX533966, KX533967 and KX533968, respectively) were obtained by PCR and subjected to functional characterization (see below). Five additional genes (TrTPS19, TrTPS17, TrTPS20, TrTPS18 and TrTPS16) clustered with linear-type diterpene synthases of the TPS-a family, but, because of the significant separation from sequences of previously characterized diterpene synthases, might also catalyze the formation of non-linear diterpenes of as yet unknown structure. While this was an interesting finding, it was not of direct interest to this investigation (involvement in the biosynthesis of kauranes or abietanes unlikely) and were therefore did not proceed with further characterizations. The translated sequences of two cDNAs (TrTPS8 (KY856995) and TrTPS7 (KY856996)) clustered with a recently discovered class I diterpene synthase of the TPS-b family from *T. wilfordii* (Hansen et al., 2017)
(Fig. 3), and were selected for functional characterization (see below). Other members from the TPS-b family were TrTPS3 (KY856993) and TrTPS4 (KY856994), which were functionally characterized (see below), and two additional transcripts (TrTPS5 and TrTPS6) that were quite short (< 600 bp) and could not be extended to a length that would have allowed functional characterization. Finally, nine members of the TPS-a family (TrTPS9, TrTPS10, TrTPS11, TrTPS12, TrTPS16, TrTPS17, TrTPS18, TrTPS19, and TrTPS20) clustered with known genes that encode linear diterpene cyclases and macrocyclases, which were not of direct interest to this study and were therefore not further characterized.

Diterpene synthase candidate genes were characterized by functional expression in engineered *E. coli* strains that provide appropriate precursors (Cyr et al., 2007). Appropriately truncated cDNAs of class II diterpene synthase candidates from *T. regelii* (lacking the plastidial targeting sequence) (Supplemental Fig. S3) were introduced into *E. coli* harboring a recombinant geranylgeranyl diphosphate synthase (GGPPS) gene from *Abies grandis* (Croteau, 2002), in combination with either the *ent*-kaurene synthase (eKS) gene from *Arabidopsis thaliana* (Yamaguchi et al., 1998) or miltiradiene synthase (MDS) gene from *Saliva miltiorrhiza* (Gao et al., 2009). Expression of TrTPS2 (candidate diterpene synthase gene from the TPS-c family) led to the production of miltiradiene when combined with MDS, but did not generate detectable products in other combinations, indicating that the gene encodes a (+)-copalyl diphosphate synthase ((+)-CPS) (Fig. 4A). The combination of TrTPS1 (also a TPS-c family member) with eKS yielded *ent*-kaurene and small amounts of *ent*-isokaurene, but no products were detected when it was expressed in other gene combinations, indicating that this gene encodes an *ent*-copalyl diphosphate synthase (ent-CPS) (Fig. 4B).

A similar modular approach was taken for characterization of the class I diterpene synthase candidates (once again lacking the plastidial targeting sequence) (Supplemental Fig. S4), involving co-expression with GGPPS (from *Abies grandis*) and either *ent*-CPS (from *Arabidopsis thaliana*), (+)-CPS (a mutant version of abietadiene synthase from *Abies grandis* that produces (+)-copalyl diphosphate) or syn-CPS (from *Oryza sativa*) (Cyr et al., 2007). *ent*-Kaurene was the most prominent reaction product (with *ent*-isokaurene as a minor side product) when TrTPS13 or TrTPS14 (TPS-e/f
family members) were expressed in combination with ent-CPS (Fig. 5A, B), indicating ent-kaurene synthase functions for these isoforms. When combined with (+)-CPS, TrTPS13 expression produced small amounts of sandaracopimaradiene and isopimaradiene, while co-expression of TrTPS13 with syn-CPS led to the formation of syn-pimara-7,15-diene and syn-stemod-13(17)-ene (Fig. 5C, D). The combination of both class II and class I diterpene synthases from Tripterygium (TrTPS2 and TrTPS13) in one modular construct resulted in the production of ent-kaurene (with smaller quantities of ent-isokaurene) (Supplemental Fig. S5), thus confirming the function of these genes in ent-kaurane diterpenoid biosynthesis. TrTPS15 (also a member of the TPS-e/f family) produced small quantities of ent-manool, syn-manool or (+)-manool when coupled with ent-CPS, syn-CPS or (+)-CPS, respectively (Fig. 5E-G). When coupled with (+)-CPS, TrTPS8 (from the TPS-b family) (Supplemental Fig. S6) produced primarily miltiradiene (Fig. 6A), along with small amounts of abietatriene presumably arising from a previously noted autoxidation (Zi and Peters, 2013). Both products were also found to be present in the medium of T. regelii adventitious root cultures (Supplemental Fig. S1). When combined with ent-CPS or syn-CPS, TrTPS8 generated small quantities of ent-manool or syn-manool, respectively (Fig. 6B, C).

TrTPS3 and TrTPS4, members of the TPS-b subfamily (Supplemental Fig. S6), did not form products in any combination with CPSs. Instead, both TrTPS3 and TrTPS4 produced mixtures of monoterpenes upon reaction with geranyl diphosphate (substrate for monoterpenene synthases), with (-)-linalool and (+)-linalool as major products (Table 2 and Supplemental Fig. S7). When reacted with linalyl diphosphate (reaction intermediate of monoterpenene synthases), TrTPS3 and TrTPS4 both formed (-)-terpineol and (+)-terpineol. In assays with linalyl diphosphate, TrTPS3, but not TrTPS4, also generated appreciable amounts (-)-limonene (~ 20 % of total products) (Table 2). TrTPS7 was expressed in the recombinant E. coli strains but was not active in any combination with copalyl diphosphate synthases. It also did not generate products from geranyl diphosphate as a substrate in in vitro assays.
DISCUSSION

Proposing a Diterpenoid Biosynthetic Pathway Based On Metabolites that Accumulate in Tripterygium Adventitious Root Cultures

In contrast to roots of mature Tripterygium plants, where diterpenoids are only very minor constituents (< 0.01 %), our adventitious root cultures secreted these metabolites at very high levels (approximately 77 % of the area under all peaks detected by HPLC-QTOF-MS in organic extracts of the culture medium). The structure and abundance of the principal diterpenoids found in our tissue cultures can thus be employed to develop hypotheses regarding the biosynthesis of abietane-type diterpenoids of Tripterygium (Fig. 2). It has previously been proposed that the two-step cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate, via (+)-copalyl diphosphate, yields miltiradiene as an abietane hydrocarbon intermediate (Hansen et al., 2017), and our data agree with this suggestion. Oxidation at C18 akin to that described for CYP720 in conifers (Ro et al., 2005; Hamberger et al., 2011; Geisler et al., 2016) would produce dehydroabietic acid (which we detected in Tripterygium root cultures) (Fig. 2). Demethylation at C4, carboxylation at C3 and hydroxylation at C14 would result in the formation of triptinin B. This metabolite was not detected in our root cultures but its methyl ether, triptinin A, was tentatively identified as a significant constituent. The tentatively identified neotriptophenolide and its glycoside, both likely derived from triptinin B and/or triptinin A, accumulated as significant byproducts in our root cultures. The lactonization of triptinin B would generate triptophenolide, which accumulated as the most abundant abietane diterpenoid in our root cultures (Fig. 2). The functionalization of the aromatic ring would then produce triptolide and, following an additional hydroxylation, tripdiolide, as the primary end products of the biosynthetic conversions detected in our adventitious root cultures.

Schemes for abietane diterpenoid biosynthesis in the genus Tripterygium have been proposed before (Kutney and Han, 1996; Kutney et al., 1981), but these were based on the available phytochemical evidence at the time and did not incorporate information about relative metabolite abundance (Fig. 1 and Supplemental Fig. S1). By adding this
new dimension, we are increasing the confidence in predictions regarding the design
principles of the pathways leading to structurally unusual diterpene triepoxides.
Because of the high abundance of diterpenoids in the medium of *Tripterygium*
adventitious root cultures, we have been able to employ simple purification protocols for
intermediates (triptophenolide) and end products (triptolide and tripdiolide). This
provides commercially unavailable substrates and products for *in vitro* enzyme assays
and, therefore, enables the functional characterization of candidate genes.

**Identifying Genes with Roles in Generating Diterpenoid Structural Diversity**

One *T. regelii* clone (TrTPS1) was characterized as encoding an ent-CPS that, in
combination with eKSs (TrTPS13 and TrTPS14), generates ent-kaurene (and very small
amounts of ent-isokaurene) (Fig. 5). These reactions are common to all vascular
plants, which require endogenous production of the derived gibberellin hormones (Zi et
al., 2014). However, ent-kaurene diterpenoids also occur as specialized metabolites in
some plant families, including the Celastraceae (comprising the genus *Tripterygium*).
The previously reported metabolites can be grouped into two major classes: four-ring
ent-kauranic acids/alcohols and five-ring oxygen bridge-containing ent-kauranes (Duan
et al., 1999; Duan et al., 2001; Tanaka et al., 2004). In the closely related species *T.
wilfordii*, recent studies also identified an ent-CPS (TwTPS3) and several isoforms of
ent-kaurene/ent-isokaurene synthase (TwTPS2, TwTPS16, TwTPS17 and TwTPS18)
(Zerbe et al., 2013; Hansen et al., 2017). Various biological activities of *Tripterygium*
ent-kauranes have been documented in *in vitro* assays (reviewed in Brinker et al.,
2007), but the *in vivo* functions remain to be elucidated. When combined with a (+)-
CPS, TrTPS13 formed sandaracopimaradiene and isopimaradiene; in combination with
a syn-CPS, TrTPS13 showed activity for the production of syn-pimara-7,15-diene and
syn-stemod-13(17)-ene (Fig. 5). We did not find a syn-CPS in *T. regelii*, nor does there
appear to be such an activity among the class II diterpene synthases of *T. wilfordii*
(Hansen et al., 2017), and the biological relevance of this finding is therefore unknown
at this time.
Genes from *T. wilfordii* were previously characterized as coding for *ent*-copal-8-ol diphosphate synthase (termed TwTPS21) or kolavenyl diphosphate synthase (TwTPS10, TwTPS14, TwTPS28) (Andersen-Ranberg et al., 2016; Hansen et al., 2017). However, the stereochemistry at C8 and C9 (8S, 9S) is the opposite of that of all labdane-type diterpenoids characterized from *Tripterygium* thus far (8R, 9R) (Duan et al., 1999; Duan et al., 2001). In our functional assays, TrTPS15 produced *ent*-manool, (+)-manool and syn-manool when combined with *ent*-CPS, (+)-CPS or syn-CPS, respectively. Interestingly, (+)-manool has the correct stereochemistry (8R, 9R) to serve as a precursor for the known labdane diterpenoids of *Tripterygium* (Duan et al., 1999; Duan et al., 2001). The other manools and manoyl oxides, generated by diterpenes synthases of *T. regelii* and *T. wilfordii*, respectively, may only be produced *in vivo* under specific environmental conditions or simply reflect enzymatic substrate promiscuity. However, the generation of structural diversity by combinations of class II and class I diterpene synthases, whether or not with recognizable *in vivo* relevance, does provide biosynthetic access to these and derived diterpenoids.

The gene coding for *T. regelii* TrTPS8 (converts (+)-copalyl diphosphate to miltiradiene, a likely intermediate in triptolide and tripdiolide biosynthesis) is an ortholog of the recently characterized TwTPS27 gene from *T. wilfordii* (Hansen et al., 2017). Based on phylogenetic analyses, Hansen et al. (2017) concluded that TwTPS27 diversified from members of the TPS-b subfamily, more specifically from terpene synthases that catalyze the formation of acyclic monoterpenes. The location of TwTPS27 on the phylogenetic tree of angiosperm terpene synthases was a surprise because all angiosperm diterpene synthases involved in abietane/labdane biosynthesis characterized until then belonged to the TPS-e/f subfamily (Chen et al., 2011). This evolutionary history also applies to TrTPS8, which is very closely related to TwTPS27.

Hansen et al. (2017) expressed TwTPS23, TwTPS24 and TwTPS26 transiently in *Nicotiana benthamiana*, and then subjected volatiles to a headspace analysis. The authors did not have authentic standards for monoterpenes and therefore could only use spectral comparisons for tentative identification. We are in the fortunate position to host a sizable repository of monoterpenes and therefore were able to perform *in vitro* assays with unequivocal results. TrTPS3 and TrTPS4 catalyzed the formation of a
mixture of (-)-linalool and (+)-linalool from geranyl diphosphate as a substrate. These result from early termination (by water capture), immediately following the formation of a linalyl cation (Supplemental Fig. S6). To test if these enzymes would be capable of catalyzing cyclization if initial water capture was avoided, we also performed assays with linalyl diphosphate as a substrate. Indeed, in these assays, TrTPS3 and TrTPS4 formed (-)-terpineol and (+)-terpineol as products (with TrTPS3 also releasing (-)-limonene), indicating that, although water capture is still the main means of terminating the catalyzed reaction, these TPSs can catalyze cyclization. To the best of our knowledge, these are the first monoterpene synthases to be unequivocally identified from *Tripterygium*.

In summary, the high abundance of diterpene synthase transcripts in *Tripterygium* adventitious root cultures facilitated the rapid cloning of candidate genes. These were functionally characterized using a suitable modular expression system (Cyr et al., 2007). Thus, our tissue cultures are an excellent experimental system for further pathway elucidation.

**Can Adventitious Root Cultures Serve as Sustainable Resources for the Production of Pharmaceutically Relevant Diterpenoids?**

Cultures of *Tripterygium* hairy roots, adventitious roots or cell suspensions, all have the advantage of accumulating abietane diterpenoids at high concentrations (Miao *et al.*, 2013; Zhu *et al.*, 2014) and, in comparison to the unsustainable harvest of roots (where the concentrations of these specialized metabolites are very low), should be considered as commercial sources. We demonstrate here that diterpenoids are secreted into the culture medium of *Tripterygium* adventitious root cultures, which significantly reduces the complexity of the matrix for extraction. Triptolide is detected as the peak with the second highest intensity (16 % of the total peak area) in our HPLC-QTOF-MS chromatograms of culture medium extracts. Furthermore, it is well separated from other medium constituents, which enabled a one-step HPLC processing to > 95 % purity (as judged by HPLC-QTOF-MS and $^1$H-NMR). In our hands, the production of triptolide in adventitious root cultures has also been reliable (grown continuously for more than two
years) and readily scalable (from 50 to 500 mL volumes within weeks). Others have scaled up *Tripterygium* cultures to 10-20 L bioreactors (Kutney et al., 1992; Miao et al., 2014), and it therefore appears that typical shortcoming of tissue culture (reliability and scalability) have already been addressed.

It is probable that triptolide levels could be further enhanced if flux through the abietane diterpenoid pathway was increased in transgenic tissue cultures, where genes involved in diterpenoid biosynthesis are already expressed at fairly high levels (Supplemental Fig. S8). One approach would be the over-expression of genes involved in providing diterpenoid precursors. A different and potentially complimentary approach would be the down-regulation of genes involved in competing pathways (e.g., reducing triterpenoid formation). We are currently investigating methods for the transformation of *Tripterygium* to enable such endeavors. Alternatively, the genes required for triptolide biosynthesis, once discovered, could be transferred to an engineered microbial strain, akin to the successful efforts to produce another diterpenoid, forskohlin (Pateraki et al., 2017). At this point in time, yields of highly functionalized plant diterpenoids in synthetic hosts have been relatively low, and plant tissue cultures would therefore seem to be a competitive option for triptolide production.

**Experimental Procedures**

**Chemicals**

Triptolide and celastrol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Dehydroabietic acid was synthesized according to a literature protocol (Gonzalez et al., 2010). Acetone was of HPLC grade (Fischer Scientific, Pittsburgh, PA, USA), ethyl acetate was ACS quality (Avantor Performance Materials Inc, Center Valley, PA, USA), ethanol was Omnisolv, (EMD Serono, Rockland, MA, USA), and acetonitrile and water were LC-MS grade (Sigma-Aldrich, St. Louis, MO, USA). CDCl$_3$ was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Adventitious Root Cultures

Young *T. regelii* Sprague & Takeda plants were purchased from Woodlanders Inc. (Aiken, SC, USA) and maintained under greenhouse conditions (illumination: 16 h day, 8 h night (250-500 μE); temperature: 24 °C day, 20 °C night; relative humidity: 45-55 %). To initiate tissue cultures, leaf material was harvested, rinsed with sterile H₂O, and surface-sterilized by soaking in 20 % (v/v) commercial bleach. Sterilized leaves were cut into 1 cm² squares and placed onto with Murashige & Skoog (MS) media with macro and micro-nutrients plus Gamborg's vitamins (Caisson, Logan, UT, USA), sucrose (20 g L⁻¹; Sigma-Aldrich, St. Louis, MO, USA), 1-naphthaleneacetic acid (0.2 mg L⁻¹; Caisson, Logan, UT, USA), 6-benzylaminopurine (1.0 mg L⁻¹; Sigma-Aldrich, St. Louis, MO, USA), phytagel (2.4 g L⁻¹; Sigma-Aldrich, St. Louis, MO, USA), and a pH adjusted to 5.8. After 4-6 weeks, callus that developed along the leaf square edges was moved to plates containing root induction medium (MS media with macro and micro-nutrients plus Gamborg's vitamins, sucrose (20 g L⁻¹), 1-naphthaleneacetic acid (1.0 mg L⁻¹), 6-benzylaminopurine (0.2 mg L⁻¹), phytagel (2.4 g L⁻¹); pH adjusted to 5.8). Developing adventitious roots were transferred to fresh plates every 4-6 weeks until they appeared strong enough for transfer to liquid medium (MS media with macro and micro-nutrients plus Gamborg's vitamins, sucrose (20 g L⁻¹), 1-naphthaleneacetic acid (1.0 mg L⁻¹), pH 5.8). Adventitious roots were partially submerged in medium (50 mL in a 250 mL Erlenmeyer flask) and maintained at 25°C, by shaking at 80 revelations per minute, in the dark, with transfer to fresh medium every two weeks. As adventitious roots grew, the size of the flask and volume of liquid medium were adjusted up to 500 mL in a 2 L Fernbach flask.

Metabolite Extraction from Adventitious Root Cultures

Solid materials from adventitious root cultures were washed with water (volume equivalent to wet weight of material), freeze-dried for 3 d (Lyph-Lock 12L, LabConco, Kansas City, MO, USA), and the resulting dry matter stored at -80°C until further use. Aliquots of 50 ± 0.5 mg were transferred to receptacles of a MM01 Dry Mill (Retsch,
Newtown, PA, USA) and further homogenized by ball shaking for 45 s at a rate of 20 shakes per second. The homogenate was then transferred to glass test tubes with teflon-lined caps. Each sample was extracted 4 times at 23°C with 5 mL acetone for 30 min (Ultrasound Bath FS30H, Fischer Scientific). After each extraction step, samples were centrifuged at 3,000 x g for 5 min and the combined supernatants collected in a separate glass test tube. The solvent was removed under reduced pressure (EZ Bio, GeneVac, Stone Ridge, NY, USA) and the remaining residue re-dissolved in 1 mL 90 % acetonitrile containing 10 μg/mL 9-anthracene carboxylic acid as internal standard. Prior to further analysis, samples were passed through syringe filters (polytetrafluoroethylene; 0.22 μM pore size) and stored at 4°C for a maximum of 2 d.

A medium aliquot of adventitious root cultures (generally 5 mL) was extracted twice with ethyl acetate (2 mL each time), by thorough mixing for 1 min at 23°C. The combined organic extracts (4 mL) were extracted against water (2 mL), transferred to a new glass vial, and the solvent was removed under reduced pressure (EZ Bio, GeneVac, Stone Ridge, NY, USA). The residue was re-dissolved in 200 μl 90 % aqueous acetonitrile containing 10 μg/mL 9-anthracene carboxylic acid as internal standard. Prior to further analysis, samples were passed through syringe filters (polypropylene; 0.22 μM pore size) and stored at 4°C for a maximum of 2 d.

**HPLC-QTOF-MS and MS/MS Data Acquisition and Method Validation**

The separation of metabolites was almost identical to that described previously (Fischedick et al., 2015). The conditions for metabolite separation were modified slightly: the initial conditions were 70 % solvent A (water with 0.1 % (v/v) formic acid) and 30 % solvent B (acetonitrile with 0.1 % (v/v) formic acid). A linear gradient (flow rate 0.6 mL/min) was used to increase solvent B to 80 % over 35 min, followed by a more rapid gradient to 95 % solvent B at 40 min. The diode array detector was set to record at 219, 254, and 424 nm, with UV/VIS spectra being recorded from 200-500 nm. Mass spectral data were obtained based on the protocols outlined previously (Fischedick et al., 2015) with the following differences: the electrospray ionization source was operated in positive polarity and MS/MS data were acquired with a collision
Data analysis was performed using the MassHunter software version B.03.01 (Agilent Technologies, Santa Clara, CA, USA). The approach for validating the quantitation of triptolide and celastrol was reported previously for various analytes in plant matrices (Cuthbertson et al., 2013) and only the relevant values are given here: recovery from adventitious roots (n = 3): 85.6 ± 5.6 % for triptolide, 96.8 ± 2.8 % for celastrol; recovery from culture medium (n = 3): 106.6 ± 3.9 % for triptolide, 86.1 ± 2.9 % for celastrol; reproducibility of extraction/detection (as relative standard deviation; n = 3): 6.5 % (interday) and 0.9 % (intraday) for triptolide, 0.5 % (interday) and 0.6 % (intraday) for celastrol; limit of detection at signal-to-noise ratio of 1 : 5 (n = 3): 0.01 ng for triptolide (MS detection) and 1.0 ng for celastrol (diode array detection at 424 nm); limit of quantitation at signal-to-noise ratio of 1 : 10 (n = 3): 0.05 for triptolide (MS detection) and 10 ng for celastrol (diode array detection at 424 nm); regression equation for calibration curve (n = 3): y = 25938x + 16475 (R² = 0.989) for triptolide (corrected for matrix effects) and y = 0.9077 x (R² = 0.999) for celastrol (matrix effects irrelevant for diode array detection); and linear range: 0.05 – 50.0 ng for triptolide and 1.0 – 2,500 ng for celastrol.

Cloning and Functional Characterization of Terpene Synthases

Raw data from several transcriptome sequencing projects previously performed with T. regelii tissues (NCBI Short Read Archive accession number SRP075639 (adventitious root cultures)) and data sets available at http://www.medplantrnaseq.org/ (roots and root cultures) were downloaded and a consensus assembly generated using the Trinity (Haas et al., 2013) and TransABySS (Robertson et al., 2010) assemblers. tBLASTn searches with peptide sequences of characterized class I and class II diterpene synthases (Supplemental Table S2) were performed against the T. regelii consensus assembly. RNA was isolated from T. regelii adventitious root cultures (harvested at 10 d after transfer to fresh medium) using the Plant RNA Purification reagent (Invitrogen, Carlsbad, CA, USA) and the RNEasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, converted to cDNA using Maxima Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA), and PCR reactions with
gene-specific primers were then employed to clone candidate diterpene synthase sequences (details in Supplemental Table S3). RACE-PCR was performed according to the manufacturer’s instructions (SMARTer-RACE 5′/3’ kit, Clontech/TaKaRa, Mountain View, CA, USA). The Gateway® cloning system (Invitrogen, Carlsbad, CA, USA) was used to insert candidate diterpene synthases into vectors of the pGGxC series for functional expression in the *E. coli* C41 (DE3) (Cyr et al., 2007). Diterpenoids were extracted directly from 50 mL cultures with an equal volume of n-hexanes (Fisher Scientific, Fair Lawn, NJ, USA). The extract was then run through silica gel 60 and magnesium sulfate columns as described elsewhere (Cyr et al., 2007). The eluents were dried under a flow of nitrogen and the residue was dissolved in 200 µl n-hexanes. Aliquots (1 µL) were injected onto an HP-5MS column (30 m length x 0.25 mm diameter; 0.25 µm film thickness; J&W Scientific, distributed through Agilent, Santa Clara, CA, USA) of a 6890N gas chromatograph (operated in splitless mode) coupled to a 5973 inert mass selective detector (Agilent, Santa Clara, CA, USA). Settings: helium as carrier gas at a flow rate of 1 mL min⁻¹; inlet temperature set to 250°C; oven program with 50°C for 1 min, first linear gradient to 300°C at 7°C min⁻¹, second linear gradient to 330°C with 20°C min⁻¹, and final hold of 5 min; transfer line set to 180°C; electron impact spectra recorded at 70 eV with MS data collection from m/z 50 to 450. Peaks were identified based on comparisons of retention time and MS fragmentation patterns with those of authentic standards. The functional evaluation of monoterpene synthases was performed according to Srividya et al. (2015).

All genes characterized as part of this study have been deposited in GenBank with the following accession numbers: KX533964 (TrTPS2); KX533965 (TrTPS1); KX533966 (TrTPS13); KX533967 (TrTPS14); KX533968 (TrTPS15), KY856993 (TrTPS3), KY856994 (TrTPS4), KY856995 (TrTPS8), and KY856996 (TrTPS7).

**ACKNOWLEDGMENTS**

This work was supported by the National Institutes of Health (award number RC2GM092561 to B.M.L. and GM076324 to R.J.P.) and Hatch funds from the Agricultural Research Center at Washington State University (to B.M.L.). The authors
thank Sean R. Johnson for generating sequence assemblies with publicly available transcriptome data sets. The authors would also like to thank Washington State University’s NMR Core Facility for access to instruments and expert support from Dr. Greg Helms.

Legends to Tables, Figures and Supplemental Data

Table 1. Metabolites detected by HPLC-QTOF-MS in the medium of _Tripterygium_ adventitious root cultures. MS/MS spectra were acquired with a fragmentation voltage of 30 eV.

Table 2. Product distribution in _in vitro_ assays with TrTPS3 and TrTPS4.

**Fig. 1.** Accumulation of abietane diterpenoids in _Tripterygium_ adventitious root cultures. HPLC-QTOF-MS traces (total ion current) obtained with ethanolic extracts of cultured roots (**A**) and culture medium (**B**). The identity of the metabolite peaks 1-13 is given in Table 1 (peak #13 (celastrol) is highlighted in blue because it is the only metabolite that does not belong to the abietane diterpenoids). Fernbach flasks with root culture photographed from below (**C**); the arm of the person holding the 4 L flask is visible at the bottom right and serves as a size comparison. Concentrations of triptolide and celastrol in cultured roots and culture medium (**D**).

**Fig. 2.** Putative biosynthetic pathway toward abietane diterpenoids in _Tripterygium_. Metabolites found in this study are boxed. The numbering of the carbons forming the abietane skeleton is indicated using dehydroabietic acid as an example. Reactions requiring multiple steps are indicated by dotted arrows. The numbering of metabolites is the same as in Fig. 1.

**Fig. 3.** Molecular phylogeny of dicot (di)terpene synthases, with an emphasis on those from the genus _Tripterygium_. Functionally characterized terpene synthases from _T. regelii_ are highlighted by grey boxes. The analysis was carried out using the Maximum Likelihood method based on a matrix-based model (Jones et al., 1992). The bootstrap
consensus tree inferred from 1,000 replications (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances, and then selecting the topology with superior log likelihood value. The analysis involved 77 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 94 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Fig. 4. Modular in vivo assay results obtained with Tripterygium class II diterpene synthase candidates of the TPS-c subfamily. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin; MDS, miltiradiene synthase (from Salvia miltiorrhiza); eKS, ent-kaurene synthase (from Arabidopsis thaliana)), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. TrTPS2 was identified as a (+)-copalyl diphosphate synthase (A), while TrTPS1 showed activity as an ent-copalyl diphosphate synthase (B).

Fig. 5. Modular in vivo assay results obtained with Tripterygium class I diterpene synthase candidates of the TPS-e/f family. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. The following gene combinations were tested: (A) ent-CPS/TrTPS13, (B) ent-CPS/TrTPS14, (C) (+)-CPS/TrTPS13, (D) syn-CPS/TrTPS13, (E) ent-CPS/TrTPS15, (F) (+)-CPS/TrTPS15, (G) syn-CPS/TrTPS15.

Fig. 6. Modular in vivo assay results obtained with TrTPS8, a class I diterpene synthase candidate of the TPS-a subfamily. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. The
following gene combinations were tested: (A) ent-CPS/TrTPS8, (B) (+)-CPS/TrTPS8, (C) syn-CPS/TrTPS8.

SUPPLEMENTAL DATA

Supplemental Fig. S1. Accumulation of abietane diterpenoids in the medium of *T. regelii* adventitious root cultures.

Supplemental Fig. S2. $^1$H-NMR spectra of abietane diterpenoids.

Supplemental Fig. S3. Sequences of *T. regelii* diterpene synthases of the TPS-c family.

Supplemental Fig. S4. Sequences of *T. regelii* diterpene synthases of the TPS-e/f family.

Supplemental Fig. S5. Sequences of *T. regelii* diterpene synthases of the TPS-b family.

Supplemental Fig. S6. Modular *in vivo* combination of *T. regelii* class II (TrTPS2) and class I (TrTPS13) diterpene synthases.

Supplemental Fig. S7. Proposed mechanism for *T. regelii* monoterpane synthases. OPP denotes the diphosphate moiety.

Supplemental Fig. S8. Comparison of expression levels of candidate diterpene synthase genes in *T. regelii* adventitious root cultures (ARC) and roots based on RNA Sequence by Expectation-Maximization analysis.

Supplemental Fig. S9. Alignment of diterpene synthase sequences included in the phylogenetic analysis.

Supplemental Table S1. Changes in triptolide and celastrol concentrations in *T. regelii* adventitious root cultures following various elicitor treatments.

Supplemental Table S2. List of dicot diterpene synthase protein sequences used in the phylogenetic analysis. *Physcomitrella patens* CPS/KS serves as outgroup.

Supplemental Table S3. List of primers used in *T. regelii* terpene synthase gene cloning.

Supplemental Methods and Data File S1. Purification and characterization of diterpenoids from *T. regelii* adventitious root cultures.
Supplemental Fig. S1. Accumulation of abietane diterpenoids in the medium of *T. regelii* adventitious root cultures. (A) HPLC-QTOF-MS chromatogram of organic extract and corresponding integration table (B). Peaks for abietane diterpenoids are indicated with arrows. (C) Detection of abietane hydrocarbon intermediates by GC-MS.

Supplemental Fig. S2. ^1^H-NMR spectra of abietane diterpenoids. (A) Triptophilinolide, (B) triptolide, (C) tripdiolide, (D) dehydroabietyllic acid.

Supplemental Fig. S3. Sequences of *T. regelii* diterpene synthases of the TPS-c family. The aspartate-rich DXDD motif is indicated by a black square.

Supplemental Fig. S4. Sequences of *T. regelii* diterpene synthases of the TPS-e/f family. The solid triangle indicates the predicted cleavage site of the plastidial targeting sequence for TrTPS13 and TrTPS14. The hollow triangle indicates the same for TrTPS15. The black square marks the aspartate-rich DDXXD motif.

Supplemental Fig. S5. Sequences of *T. regelii* diterpene synthases of the TPS-b family. The solid triangle indicates the predicted cleavage site of the plastidial targeting sequence for TrTPS8. The patterned and hollow triangles indicate the same for TrTPS3/TrTPS4 and TrTPS7, respectively. The black square marks the aspartate-rich DDXXD motif.

Supplemental Fig. S6. Modular *in vivo* combination of *T. regelii* class II (TrTPS2) and class I (TrTPS13) diterpene synthases. (A) plasmid constructs, (B) chromatogram of products, (C) mass spectra of peaks shown in (B), (D) mass spectra of authentic standards.

Supplemental Fig. S7. Proposed mechanism for *T. regelii* monoterpen synthases. OPP denotes the diphosphate moiety.
Supplemental Fig. S8. Comparison of expression levels of candidate diterpene synthase genes in *T. regelii* adventitious root cultures (ARC) and roots based on RNA Sequence by Expectation-Maximization analysis.

Supplemental Fig. S9. Alignment of diterpene synthase sequences included in the phylogenetic analysis.

Supplemental Table S1. Changes in triptolide and celastrol concentrations in *T. regelii* adventitious root cultures following various elicitor treatments.

Supplemental Table S2. List of dicot diterpene synthase protein sequences used in the phylogenetic analysis. *Physcomitrella patens* CPS/KS serves as outgroup.

Supplemental Table S3. List of primers used in *T. regelii* terpene synthase gene cloning.

Supplemental Methods and Data File S1. Purification and characterization of diterpenoids from *T. regelii* adventitious root cultures.
References


Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J 66: 212-229


Fischedick JT, Johnson SR, Ketchum REB, Croteau RB, Lange BM (2015) NMR spectroscopic search module for Spektraris, an online resource for plant natural product identification - Taxane diterpenoids from Taxus x media cell suspension cultures as a case study. Phytochemistry 113: 87-95


Helmstädtener A (2013) Tripterygium wilfordii Hook f - how a traditional Taiwanese medicinal plant found its way to the West. Pharmazie 68: 643-646


Downloaded from on August 25, 2017 - Published by www.plantphysiol.org
Copyright © 2017 American Society of Plant Biologists. All rights reserved.
Table 1. Metabolites detected by HPLC-QTOF-MS in the medium of *Tripterygium* adventitious root cultures. MS/MS spectra were acquired with a fragmentation voltage of 30 eV.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>RT</th>
<th>Formula</th>
<th>[M+H]^+</th>
<th>Error</th>
<th>MS/MS signal patterns</th>
<th>NMR-confirmed</th>
<th>Auth. std. confirmed</th>
<th>Tentative ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tripdiolide (1)</td>
<td>1.8</td>
<td>C20H24O7</td>
<td>359.1489</td>
<td>0.82</td>
<td>359 (100), 171 (45.1), 157 (60.3), 155 (59.7), 143 (54.3), 129 (44.8), 128 (51.9), 105 (50.0)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triptolide (2)</td>
<td>4.6</td>
<td>C20H24O6</td>
<td>361.1646</td>
<td>0.31</td>
<td>361 (100), 157 (45.4), 145 (54.9), 143 (66.0), 142 (42.2), 131 (42.8), 119 (30.3), 105 (53.5)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-O-β-D-Glucopyranosyl neotriptophenolide (3)</td>
<td>6.9</td>
<td>C27H36O9</td>
<td>505.2432</td>
<td>0.13</td>
<td>343.2 (100), 301 (21.8), 297.2 (32.7), 255 (60.2), 205 (25.6), 179 (18.6), 163 (13.7), 127 (15.2)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diterpenoid (unknown) (4)</td>
<td>9.4</td>
<td>C20H22O6</td>
<td>359.1489</td>
<td>3.66</td>
<td>359 (100), 171 (14.5), 145 (19.2), 143 (27.8), 131 (15.8), 128 (13.0), 119 (13.5), 105 (26.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diterpenoid (unknown) (5)</td>
<td>10.6</td>
<td>C21H26O3</td>
<td>327.1955</td>
<td>5.16</td>
<td>203 (45.4), 161 (100), 147 (65.9), 135 (52.4), 133 (35.3), 131 (44.2), 111 (44.0), 105 (91.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neotriptophenolide (6)</td>
<td>13.3</td>
<td>C21H26O4</td>
<td>343.1904</td>
<td>0.48</td>
<td>283 (21.5), 237 (100), 223 (28.6), 222 (52.4), 209 (20.7), 197 (24.4), 185 (41.6), 111 (22.1)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Diterpenoid (unknown) (7)</td>
<td>14.9</td>
<td>C21H26O4</td>
<td>343.1904</td>
<td>2.29</td>
<td>297 (24.5), 257 (22.7), 255 (100), 243 (22.9), 240 (42.1), 205 (62.8), 177 (28.6), 163 (57.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triptophenolide (8)</td>
<td>15.2</td>
<td>C20H24O3</td>
<td>313.1798</td>
<td>0.51</td>
<td>253 (18.7), 225 (100), 197 (19.1), 185 (23.5), 183 (47.8), 181 (25.8), 171 (25.8), 147 (22.2)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydroabietic acid (9)</td>
<td>18.1</td>
<td>C20H28O2</td>
<td>301.2162</td>
<td>2.48</td>
<td>215 (90.4), 175 (61.3), 173 (71.2), 171 (60.2), 159 (68.7), 147 (94.9), 145 (51.7), 133 (100)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triptophenolide methyl ether (10)</td>
<td>18.8</td>
<td>C21H26O3</td>
<td>327.1982</td>
<td>1.13</td>
<td>239 (100), 225 (24.3), 224 (28.9), 207 (24.9), 197 (43.8), 185 (32.4), 161 (26), 147 (40.7)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triptinin A (11)</td>
<td>19.1</td>
<td>C21H28O3</td>
<td>329.2111</td>
<td>1.35</td>
<td>255 (35.1), 237 (28.5), 189 (27.7), 175 (33.8), 171.8 (42.4), 149 (100), 147 (51.3), 133 (52.3)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diterpenoid (unknown) (12)</td>
<td>21.9</td>
<td>C21H30O2</td>
<td>315.2324</td>
<td>1.64</td>
<td>229.2 (56.6), 189 (43.2), 187 (65.1), 185 (64.0), 173 (46.4), 161 (100), 159 (41), 147 (73.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celastrol (13)</td>
<td>23.0</td>
<td>C29H38O4</td>
<td>451.2843</td>
<td>1.74</td>
<td>215 (10.3), 201 (100), 200 (5.2)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Product distribution in *in vitro* assays with TrTPS3 and TrTPS4.

<table>
<thead>
<tr>
<th>Monoterpene product</th>
<th>Retention time [min]</th>
<th>Geranyl diphosphate as substrate [% of total]</th>
<th>Linalyl diphosphate as substrate [% of total]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TrTPS3</td>
<td>TrTPS4</td>
</tr>
<tr>
<td>Myrcene</td>
<td>17.135</td>
<td>-</td>
<td>4.39</td>
</tr>
<tr>
<td>(-)-Limonene</td>
<td>20.567</td>
<td>7.77</td>
<td>-</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td>20.823</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>23.734</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(-)-Linalool</td>
<td>32.171</td>
<td>35.60</td>
<td>40.38</td>
</tr>
<tr>
<td>(+)-Linalool</td>
<td>32.327</td>
<td>44.77</td>
<td>46.04</td>
</tr>
<tr>
<td>(-)-Terpineol</td>
<td>40.083</td>
<td>7.07</td>
<td>4.83</td>
</tr>
<tr>
<td>(+)-Terpineol</td>
<td>40.359</td>
<td>4.78</td>
<td>4.35</td>
</tr>
</tbody>
</table>
**Fig. 1.** Accumulation of abietane diterpenoids in *Tripterygium* adventitious root cultures. HPLC-QTOF-MS traces (total ion current) obtained with ethanolic extracts of cultured roots (A) and culture medium (B). The identity of the metabolite peaks 1-13 is given in Table 1 (peak #13 (celastrol) is highlighted in blue because it is the only metabolite that does not belong to the abietane diterpenoids). Fernbach flasks with root culture photographed from below (C); the arm of the person holding the 4 L flask is visible at the bottom right and serves as a size comparison. Concentrations of triptolide and celastrol in cultured roots and culture medium (D).
Fig. 2. Putative biosynthetic pathway toward abietane diterpenoids in *Tripterygium*. Metabolites found in this study are boxed. The numbering of the carbons forming the abietane skeleton is indicated using dehydroabietic acid as an example. Reactions requiring multiple steps are indicated by dotted arrows. The numbering of metabolites is the same as in Fig. 1.
Fig. 3. Molecular phylogeny of dicot (di)terpene synthases, with an emphasis on those from the genus Tripterygium. Functionally characterized terpene synthases from T. regelii are highlighted by grey boxes. The analysis was carried out using the Maximum Likelihood method based on a matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 1,000 replications (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances, and then selecting the topology with superior log likelihood value. The analysis involved 77 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 94 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).
Fig. 4. Modular in vivo assay results obtained with *Tripterygium* class II diterpene synthase candidates of the TPS-c subfamily. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin; MDS, miltiradiene synthase (from *Salvia miltiorrhiza*); eKS, ent-kaurene synthase (from *Arabidopsis thaliana*)), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. TrTPS2 was identified as a (+)-copalyl diphosphate synthase (A), while TrTPS1 showed activity as an ent-copalyl diphosphate synthase (B).
Fig. 5. Modular in vivo assay results obtained with Tripterygium class I diterpene synthase candidates of the TPS-e/f family. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. The following gene combinations were tested: (A) ent-CPS/TrTPS13, (B) ent-CPS/TrTPS14, (C) (+)-CPS/TrTPS13, (D) syn-CPS/TrTPS13, (E) ent-CPS/TrTPS15, (F) (+)-CPS/TrTPS15, (G) syn-CPS/TrTPS15.
Fig. 6. Modular in vivo assay results obtained with TrTPS8, a class I diterpene synthase candidate of the TPS-a subfamily. Left to right: constructs (selectable markers in gray boxes; Chlor, chloramphenicol; Carb, carbenicillin), GC-MS chromatograms, mass spectra of assay products and mass spectra of authentic standards. The following gene combinations were tested: (A) ent-CPS/TrTPS8, (B) (+)-CPS/TrTPS8, (C) syn-CPS/TrTPS8.
Parse Citations


Chen F, Tholl D, Bohlm ann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J 66: 212-229


Fischedick JT, Johnson SR, Ketchum REB, Croteau RB, Lange BM (2015) NMR spectroscopic search module for Spektraris, an online resource for plant natural product identification - Taxane diterpenoids from Taxus x media cell suspension cultures as a case study. Phytochemistry 113: 87-95


