Tripterygium wilfordii cytochrome P450s catalyze the methyl shift and epoxidations in the biosynthesis of triptonide

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Published in:
Nature Communications

DOI:
10.1038/s41467-022-32667-5

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

Document license:
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Citation for published version (APA):
Hansen, N. L., Kjaerulff, L., Heck, Q. K., Forman, V., Stærk, D., Møller, B. L., & Andersen-Ranberg, J. (2022). Tripterygium wilfordii cytochrome P450s catalyze the methyl shift and epoxidations in the biosynthesis of triptonide. Nature Communications, 13, [5011]. https://doi.org/10.1038/s41467-022-32667-5
**Tripterygium wilfordii** cytochrome P450s catalyze the methyl shift and epoxidations in the biosynthesis of triptonide

The diterpenoid triepoxides triptolide and triptonide from *Tripterygium wilfordii* (thunder god wine) exhibit unique bioactivities with potential uses in disease treatment and as a non-hormonal male contraceptives. Here, we show that cytochrome P450s (CYPs) from the CYP71BE subfamily catalyze an unprecedented 18(4→3) methyl shift required for biosynthesis of the abeo-abietane core structure present in diterpenoid triepoxides and in several other plant diterpenoids. In combination with two CYPs of the CYP82D subfamily, four CYPs from *T. wilfordii* are shown to constitute the minimal set of biosynthetic genes that enables triptonide biosynthesis using *Nicotiana benthamiana* and *Saccharomyces cerevisiae* as heterologous hosts. In addition, co-expression of a specific *T. wilfordii* cytochrome b₅ (*Tw* cyt b₅-A) increases tripptonide output more than 9-fold in *S. cerevisiae* and affords isolation and structure elucidation by NMR spectroscopic analyses of 18 diterpenoids, providing insights into the biosynthesis of diterpenoid triepoxides. Our findings pave the way for diterpenoid triepoxide production via fermentation.

The Chinese medicinal plant *Tripterygium wilfordii* (léi gōng téng, Thunder god wine) is currently the exclusive source of the high value diterpenoids triptolide (1) and triptonide (2). These unique triepoxide diterpenoids (Fig. 1) have multiple applications as exemplified by the use of triptolide as the active component in new non-lethal rodent pest management products and the recent identification of triptonide as a promising non-hormonal male contraceptive agent that shows high efficacy, being reversible and safe. Biochemical analyses show that triptonide targets one of the final steps in sperm development resulting in loss of the motility required for egg fertilization and consequently reversible male infertility. Widespread adoption of these novel applications for triepoxide diterpenoids is restricted by the high cost associated with triptolide extraction and purification from *T. wilfordii* or from established plant tissue cultures. The establishment of a *Saccharomyces cerevisiae* production platform for *T. wilfordii* derived triepoxide diterpenoids has been hampered by insufficient knowledge about the biosynthetic pathway operating in the host plant.

Diterpenoid biosynthesis in plants is initiated by carbocation mediated carbon rearrangement of geranylgeranyl diphosphate (GGPP) into labdane type diterpenoids catalyzed by diterpene synthases (dTTPS). In contrast to the canonical labdane type diterpenoids, *T. wilfordii* triepoxides harbors an unusual 18(4→3) abeo-abietane core structure (Fig. 1a). No dTTPS has been linked to the formation of the abeo-abietane diterpene backbone directly from GGPP and previous studies support that the triepoxide diterpenoids found in *T. wilfordii* are derived from the abietane diterpene miltiradiene. The biosynthesis of triptonide and triptolide must include enzymes that accept miltiradiene as a substrate, and catalyze reactions that can account for the unique positioning of the methyl groups on the A-ring of *T. wilfordii* triepoxides.
Here, we present the identification and characterization of a suite of *T. wilfordii* cytochrome P450s (CYPs) that in the heterologous hosts *N. benthamiana* and *S. cerevisiae* catalyze the formation of triptonide (2) from miltiradiene (3). To shed light on the biosynthetic processes leading to triptonide accumulation, 13 hitherto undescribed and 5 previously described diterpenoids are isolated and structure elucidated by NMR spectroscopy (Supplementary Figs. 8–53 and Supplementary Tables 1–19). In *S. cerevisiae*, the flux of diterpenoid through the cascade of orchestrated CYP oxidations is increased by co-expression of a gene encoding a specific cytochrome b5 (cytb5). The functional expression of *TuCYPs* and *TwCYPs* in *S. cerevisiae* constitute the foundation for the proof-of-concept strain for fermentation-based production of *T. wilfordii* derived triepoxide diterpenoids.

### Results

**Identification of *TuCYP82s* involved in miltiradiene oxygenation**

To identify candidate CYPs capable of catalyzing oxygenation of diterpenes in *T. wilfordii*, homology searches were performed in publicly available *T. wilfordii* RNA sequencing data deposits (Supplementary Table 20). More than 61 plant CYPs mainly from the CYP71 and CYP85 clans have been shown to utilize diterpenes as substrate[s] (Supplementary Fig. 1) and genes encoding CYPs from these two clans were used as queries in the homology-based search for candidate genes. In total, 68 candidate *TuCYP*-encoding transcripts (Supplementary Table 21) were identified and isolated from cDNA derived from root, stem and leaf tissue of *T. wilfordii*. Three of these genes have been previously shown to be involved in terpenoid biosynthesis catalyzing key steps in the biosynthetic pathway of celastrol, a high value triterpenoid found in root extracts of *T. wilfordii*.[12]

For *in planta* characterization of the *TuCYPs*, each of the encoding genes were co-expressed separately in *N. benthamiana* plants[13] together with *CfTPS1* and *CfTPS3*, that catalyze the formation of 3[14], and with GPP booster genes[15]. Combined, these are denoted as the miltiradiene biosynthetic genes. Metabolite extracts of the *N. benthamiana* leaf discs were analyzed by GC-MS and LC-qTOF-MS. Of the 68 candidate *TuCYPs* co-expressed individually with the miltiradiene biosynthetic genes, co-expression of *TuCYP82D274* resulted in the depletion of 3 with an accompanied appearance of 14-hydroxy-dehydroabietadiene (5) (Fig. 2, and Supplementary Note 1), 3-epi-triptobenzene B (13) (Supplementary Fig. 29 and Table 9) and a number of additional compounds with monoisotopic masses (detected by LC-qTOF-MS) corresponding to oxygenated diterpenoids (Supplementary Fig. 2). Metabolites 5 and 13 have previously been isolated from *T. wilfordii* root tissue or from tissue cultures along with 1 and 2.[17]

To assess whether enzymes native to *N. benthamiana* contributed to the turnover of miltiradiene-derived diterpenoids, we established an *S. cerevisiae* strain (NV50) capable of producing high levels of miltiradiene. Furthermore, to support downstream CYP functionality, the gene encoding CYP reductase *TuPOR1* (Supplementary Table 23) identified by BLAST searches in *T. wilfordii* transcriptomes, was integrated into the *S. cerevisiae* genome along with the *TuCYPs*. Interestingly, when *TuCYP82D274* was genome integrated in the established *S. cerevisiae* strain, accumulation of 5 but not 13 was observed (Supplementary Fig. 2d), demonstrating consensus for formation of only 5 when comparing the plant and microbial host systems. The products specifically formed in the plant host could reflect intrinsic properties, such as the activity of...
endogenous enzymes, affecting the direct biosynthetic products of the co-expressed enzymes.

**TuwCYP71BE86 and TuwCYP71BE85 oxygenate the A-ring of 14-hydroxy-dehydroabietadiene resulting in a C4→C3 methyl shift and lactone formation**

We hypothesized that oxygenation of the A-ring of 3 could facilitate the 18(4→3) abeo-abietaen methyl shift. Previously, CYPs from the CYP701A, CYP99A, CYP71Z, CYP71BE, and CYP71D subfamilies have been shown to be capable of oxygenating the A-ring of labdane type diterpenoids (Fig. 1 and Supplementary Fig. 1)\(^{10}\). CYP71D381 from Coleus forskohlii has been shown to oxygenate C-2 and C-20 of 13R-manoxy oxide when heterologous produced in *N. benthamiana*\(^{15}\), but to our knowledge, isolation of neither 2-hydroxy-13R-manoxy oxide nor 20-hydroxy-13R-manoxy oxide from the host plant has been reported\(^{20}\). On the other hand, a number of abietane and 18(4→3) abeo-abietane diterpenoids have been isolated from *C. forskohlii* (Fig. 1a)\(^{20}\).

We therefore hypothesized that CYP71D381 possibly accepts 3 and that members of the CYP71D subfamily could be involved in catalysis of reactions leading to the 18(4→3) methyl shift found in abietanes of both *C. forskohlii* and *T. wilfordii*. Accordingly, we tested whether 3 was a substrate for CYP71D381. Co-expression of abietadiene biosynthetic genes\(^{14}\) and CYP71D381 in *N. benthamiana* resulted in new metabolites detected in leaf extracts by LC-qTOF-MS. In the mass spectra representing these new metabolites, the monoisotopic masses of likely parental ions supported the identification (<5 ppm) of oxygenated diterpenoids (Supplementary Fig. 3), showing that CYP71D381 can efficiently oxygenate 3 in planta. With this finding we searched the TuCYP library for CYP71D381 homologs and found four CYP-encoding genes of the CYP71BE subfamily. Recent expansion of the CYP71D subfamily by the identification of new CYP genes has caused the CYP71D subfamily to gain a phylogenetic overlap with the CYP71BE subfamily (Supplementary Fig. 1). These four CYP71BE subfamily encoding genes were selected for further studies.

To test for turnover of 3, the four candidates *TuwCYP71BE83, TuwCYP71BE84, TuwCYP71BE85*, and *TuwCYP71BE86* were co-expressed individually in *N. benthamiana*, with the miatiadiene biosynthetic genes. Expression of *TuwCYP71BE85* and *TuwCYP71BE86* resulted in accumulation of oxygenated diterpenoids, with a larger number of new metabolites observed with *TuwCYP71BE86* expression in comparison to *TuwCYP71BE85* (Supplementary Fig. 3). We then tested whether the biosynthetic products of *TuwCYP72D274* could be utilized by *TuwCYP71BE85 or TuwCYP71BE86* by co-expressing each of these genes together with *TuwCYP72D274*. LC-qTOF-MS analysis of *N. benthamiana* leaf extracts revealed that co-expression of either *TuwCYP71BE85* or *TuwCYP71BE86* with *TuwCYP72D274* resulted in the accumulation of 13 in addition to other polyoxygenated diterpenoids (Fig. 2 and Supplementary Fig. 4). In leaves expressing *TuwCYP71BE86*, we also observed formation of low amounts of additional metabolites, the structures of which were identified by NMR analyses or by comparison to authentic standards. One of these were identified as triptophenolide (8) (Fig. 2), a putative intermediate in the triptolide pathway that similar to compound 1 and 2 contains the 18(4→3) abeo-abietaen core structure, and
the lactone ring at the A-ring (Fig. 1b). Two other metabolites with similar core structures were 18(S)+3-abeo-abietatrien-14,18-diol (6) and 14-hydroxy-18(S)+3-abeo-abietatrien-18-ol (7) (Fig. 2a, Supplementary Figs. 11–16 and Supplementary Tables 3–4). These results prompted us to co-express TwCYP71BE85 and TwCYP71BE86 in N. benthamiana leaves. Here we observed a significant increase in the accumulation of 8 (Fig. 2b, Supplementary Fig. 4), suggesting that both encoded enzymes can partake in the methyl rearrangement of the abietane core structure and in formation of the lactone moiety of 8.

Similar to the observations in the in planta expression host, co-expression of TwCYP82D274, TwCYP71BE85, TwCYP71BE86 and TwPOR1 in a miltiriadiene producing strain of S. cerevisiae resulted in accumulation of 8 (Fig. 2c). However, in contrast to the results obtained using the N. benthamiana system, co-expression of TwCYP82D274 and TwCYP71BE86 did not result in production of 8 in S. cerevisiae. This supports that TwCYP71BE86 upon expression in N. benthamiana as well as in S. cerevisiae catalyzes the formation of the 18(S)+3-abeo-abietate backbone and that TwCYP71BE85 expression in S. cerevisiae is required for catalysis of lactone ring formation and thus for formation of 8 (Fig. 2b, c).

**TwCyp72-A enhances the capacity of TwCYPs to catalyze multiple oxygenations of their diterpenoid substrates**

It is well established that cyt b5 may serve as an additional electron donor in CYP catalyzed reactions. To possibly improve the yield of the TwCYP products obtained in S. cerevisiae, the genes encoding six different TwCyp72s were expressed individually in S. cerevisiae strains together with TwCYPs needed for biosynthesis of 8. Co-expression of TwCyp72-A resulted in a substantial increase of 8 in the S. cerevisiae extracts, while expression of the other TwCyp72s did not have the same effect (Supplementary Fig. 5). Co-expression of TwCyp72-A with the different combinations of TwCYPs both in N. benthamiana and in S. cerevisiae (Fig. 2) revealed that the levels of CYP products were predominantly improved in the fungal host. Quantitative data for the accumulation of miltiriadiene-derived diterpenoids by transient expression in leaves of N. benthamiana showed substantially higher variability across replicates when compared to production in engineered S. cerevisiae. This is consistent with the variability observed in other N. benthamiana heterologous biosynthesis studies. Still, it is to be noticed that co-expression of TwCyp72-A and TwCYP82D274 in N. benthamiana increased the accumulation of 8 when compared to N. benthamiana only expressing TwCYP82D274 alone. The opposite was observed in relation to the amount of 6, 7 and 8 (Fig. 2b) detected in leaf extracts co-expressing TwCYP82D274 and TwCYP71BE86, in which TwCyp72-A co-expression had a negative impact of accumulation of these compounds. This observation renders it possible that presence of TwCyp72-A effect the catalytic rate of TwCYP82D274 and TwCYP71BE86, differentially in planta. In contrast, the level of all intermediates identified in the S. cerevisiae extracts increased with TwCyp72-A co-expression, with the most pronounced effect seen on the levels of intermediates associated with expression of TwCYP71BE86 (Fig. 2c).

**TwCYP82D213 completes the biosynthetic pathway for triptonide**

The uniqueness of *T. wilfordii* triptonide diterpenoids as pharmaceutical agents are attributed to the distinct configuration of epoxides on the B- and C-rings (Fig. 1a). These epoxides are essential for bioactivity, e.g., anticancer activity of triptonide towards multiple cancer cell lines. CYPs have previously been shown to catalyze epoxide formation in plant specialized metabolism. TwCYP82D274 catalyzed oxygenation of the C-ring in 3, resulting in accumulation of 5, but also oxygenations resulting in the accumulation of other miltiriadiene derived compounds with more than two oxygens (Supplementary Fig. 2). Accordingly, we hypothesized that TwCYP82D274 in combination with other CYP82D homologs identified from *T. wilfordii* cDNA could catalyze oxygenations leading to the formation of the epoxides on the B- and C-ring of 2. Among the collection of TwCYP82D encoding genes isolated from *T. wilfordii* cDNA (Supplementary Table 21), TwCYP82D213 was selected and co-expressed with TwCYP82D274, TwCYP71BE86 and TwCYP71BE85 in N. benthamiana and subsequently in S. cerevisiae. In both expression hosts, co-expression of these four TwCYP encoding genes resulted in the accumulation of 2 (Fig. 2).

Based on these findings we conclude that the four TwCYPs together with the miltiriadiene biosynthetic genes constitute a minimal set of triptonide biosynthetic genes in *N. benthamiana* and *S. cerevisiae* expression hosts (Figs. 2 and 3).

**The route towards triepoxide formation by TwCYP catalyzed oxygenations in two orthologous biosynthesis hosts**

A number of miltiriadiene derived products including compounds with monoisotopic masses corresponding to glucosides and glutathione diterpenoid conjugates were identified by LC-qTOF-MS analysis of extracts of *N. benthamiana* leaves expressing the triptonide biosynthetic genes (Supplementary Fig. 6, Supplementary Table 24). Glucosylation and glutathionylation of oxygenated terpenoids heterologously produced in *N. benthamiana* tissues have previously been observed and their accumulation is likely caused by native *N. benthamiana* glucosyltransferases and glutathione S-transferases.

From the engineered biosynthetic hosts, 23 GGPP derived compounds were identified, including 8 which were structurally elucidated by use of 1D and 2D NMR spectroscopy in this work (Supplementary Figs. 9–53, Supplementary Table 1–20). Beside isocopal-13(16)-en-3,12,15-triol (19), labda-8(17),13E–15-O-acetate-dien-3-ol (20), 14,15-epoxygeranylgeraniol (21), and trimethylcyclohexane-11,14-diolgeraniol (22) (Supplementary Note 3, Supplementary Figs. 21–53 and Supplementary Tables 15–19), all compounds (including the lactam-diterpenoid (23) (Supplementary Note 2, Supplementary Figs. 31–53 and Supplementary Table 19) were considered to be derived from 3 (see below). Of these, 2, 3, 8, and triptolobenzene 1 (16) have previously been isolated from species within the *Tripterygium* genus (Supplementary Table 1). Furthermore, 4-epi-triptolobenzene J (14) and 4-epi-triptquinone C (18), diastereomers of compounds found in *Tripterygium* species were identified. Hence, a substantial fraction of the molecular diversity of diterpenoid compounds present in *Tripterygium* spp. can be found in the engineered *S. cerevisiae* strain (NJV111, Supplementary Table 28) extracts. This demonstrates that the concerted enzymatic capacity of only four TwCYP enzymes enables biosynthesis of a number of abietane/abeo-abietane compounds that constitute a major part of the diterpenoid compound diversity found in the *Tripterygium* species.

**Alternative biosynthetic routes for triepoxide aboe-abietane diterpenoids**

In previous work, it has been proposed that dihydroabietic acid is an intermediate in the biosynthetic pathway for aboe-abietanes including the triepoxide terpenoids from *T. wilfordii*. A possible route for dihydroabietic acid biosynthesis in *T. wilfordii* could be through *TwCYP72BB70* catalyzed oxygenation of miltiriadiene and a subsequent spontaneous oxidation. Here we find that CYPs from the CYP71BE and CYP82D subfamilies are sufficient for triepoxide biosynthesis in both *N. benthamiana* and *S. cerevisiae*. Furthermore, none of the miltiriadiene-derived compounds identified here carried a carbonyl group at C-18.

**Genomic organization of triptonide specific TwCYP genes from *T. wilfordii***

Recently a high quality chromosome level genome of *T. wilfordii* have become available. To our surprise genes from the CYP82D and...
CYP71BE subfamilies could not be identified by BLAST searches on the T. wilfordii RNA library based on the genome annotation. However, BLAST searches with the triptonide biosynthetic genes as queries on the assembled genome enabled us to identify the genome position of all (Supplementary Table 23). Interestingly, similar to TuTPS9 and TuTPS27, close homologs of TuCYP82D274 and TuCYP71BE86 were found in tandem repeats on chromosome CM023879 and CM23888, respectively (Fig. 1c). The genes encoding TuTPS9 and TuTPS27 involved in miltiradiene biosynthesis3 were identified positioned approximately 3 Mbp downstream from the TuCYP71BE86 tandem repeats (Supplementary Table 23). In contrast, the genome location of, the majority of genes enabling heterologous triptonide biosynthesis reveal that they are situated on different chromosomes in the T. wilfordii genome (Fig. 1c).

S. cerevisiae as a heterologous fermentation host for triptonide production

Co-expression of the triptonide biosynthetic genes including Tucytb7-A in S. cerevisiae (NJV11.L11) resulted in 0.081 (n = 3, SD: 0.004) mg triptonide/L in 0.5 mL cultures using a 96-well plate as fermentation vessel. Substantial quantities of other GGPP derived compounds were also identified as being produced in the triptonide biosynthesizing strains. It remains to be determined which of these may function as intermediates in the triptonide biosynthesis pathway or as shunt products arising from an inefficient, imbalanced or incomplete triptonide pathway (see below).

Based on the established triptonide biosynthetic pathway, we sought to optimize the biosynthetic output by stable integration of additional copies of the triptonide biosynthetic genes in the S. cerevisiae genome. Following successful integration of one additional copy of the four TuCYP encoding genes, the yield of triptonide doubled to 0.20 (n = 3, SD: 0.06) mg triptonide/L in S. cerevisiae (NJV 8.15) extracts, making it our elite strain. To explore the use of S. cerevisiae as a production host for 2, the elite strain was grown in a 1 L fed-batch fermentation with daily monitoring of OD600 and quantification of 8 and 2. The level of 8 reached a maximum of 2.4 mg/L on day 3, whereas the highest level of 3.79 mg/L of 2 was at day 7 (Fig. 3). The reason for the observed differences between the accumulation of the compounds over time is unclear. It could be speculated that 8 is a rate limiting intermediate in the triptonide biosynthetic pathway. OD600 level at the end of the fermentation was high as a result of considerable water evaporation occurring during the fermentation run. Still, the fed-batch fermentation of NJV8.5 enabled us to obtain production titers supporting proof-of-concept for S. cerevisiae fermentation-based production of 2.

Discussion

By co-expression of T. wilfordii biosynthetic genes in two orthogonal heterologous host organisms, we demonstrate that four genes from the CYP71BE and the CYP82D subfamilies constitute a minimal set of genes required for formation of triptonide (2) from miltiradiene (3).

The distinct nature of the two chosen production systems minimizes the likelihood of endogenous enzyme activities in the heterologous hosts being contributors to the formation of 2. Production in S. cerevisiae was more easily scaled and offered a cleaner background for purification of the intermediates selected for NMR analysis and structure elucidation.

In prior work, a majority of the functionally characterized genes from the CYP82D subfamily have been associated with enzymes catalyzing steps in flavonoid biosynthesis10. No gene from this subfamily has to our knowledge been shown to be involved in oxygenation of diterpenoids11. A reaction similar to the one catalyzed by TuCYP82D274 is carried out by SmCYP76AHI1 and homologs from Lamiaceae species resulting in formation of 11-hydroxy-dehydroabietadiene from 3 as demonstrated by expression studies in S. cerevisiae2. However, in many cases it remains to be determined whether conversion of 3 to dehydroabietadiene (4) is a spontaneous reaction or whether this happens in conjunction with oxidation of 3 resulting in aromatic ring formation (Fig. 3).

The production of CYP71BE86 was shown to catalyze an unprecedented 18(4→3) abeo-abietane methyl shift within the abietane type scaffold found in 3, 4 and 5 when its encoding gene was expressed in N. benthamiana as well as in S. cerevisiae. This demonstrates that TuCYP71BE86 catalyzes a carbon rearrangement of labdane type diterpenoids, a highly unusual CYP reaction not previously assigned to any identified plant CYP. Interestingly, EICYP71D445 from the phylogenetically overlapping subfamily (Fig. 1b, Supplementary Fig. 1) has been shown in combination with EICYP726A27 and ElADH1 to catalyze oxygenations facilitating carbon rearrangement of its macrocyclic diterpenoid substrate possibly via an aldol reaction12. SmCYP71D375 from the same CYP family has been shown to catalyze the formation of the heterocycle in Tanshinone II A from miitilone possibly via a P450 mediated carbocyclization reaction mechanism13. Thus, three plant CYPs from the CYP71D/BE subfamily have been shown to be involved in rearrangement of diterpenoid core structures.

Carbocyclation mediated carbon rearrangements are the common mechanism in terpenoid synthase (TPS) catalyzed formation of cyclic terpenoids from prenyldiphosphates14. In contrast, the reported additional ability of plant CYPs to catalyze the formation of carbocyclations mediating terpenoid carbon rearrangements opens up a new route to the formation of complex natural products. Thus, the formation of S(12)-oxa-3(11)-cycloartenone upon heterologous expression of TbcYP725A from Taxus brevifolia in N. benthamiana, has been argued to be the result of a carbocyclation mediated carbon rearrangement of taxadiene. TbcYP725A is part of the CYP85 clan. From the same clan, detailed studies of members of the CYP85A subfamily support that they catalyze ring-contraction of ent-kaurenoic acid via formation of a carbocycle and a pinacol type rearrangement. The bacterium Streptomyces arenace synthesize the terpenoid pentanolactone. The final step of its biosynthesis proceeds via a CYP16IC catalyzed formation of a carbocation, resulting in a Wagner-Meerwein type methyl shift and formation of a cyclo-pentene moiety. A similar catalytic mechanism has to our knowledge never been reported for plant CYPs. CYP16IC2 is distantly related to TuCYP71BE86 but renders it possible that TuCYP71BE86 catalyzes the 18(4→3) abeo-abietane methyl shift using a Wagner-Meerwein type reaction mechanism (Fig. 4b).

This is further supported by the observation that the compounds 13, 6, 8, 18 R(4→3) abeo-abietatrien-19-ene-14,18,20-triol (9), 18 R(4→3) abeo-abietatrien-14,18-diol (10), 18 S(4→3) abeo-abietatrien-14,18-diol (11), 18 S(4→3) abeo-abietatrien-14,18,20-triol

![Fig. 3 | Accumulation in fed-batch fermentation. The accumulation of 8 (dotted black) and 2 (solid black) from NVJ8.5 (Supplementary Table 28) grown in a fed-batch fermentation. Density of the S. cerevisiae culture was based on the optical density of daily culture samples.](https://doi.org/10.1038/s41467-022-32667-5)
18(4→3) abeo-abietatrien-14,18,20-triol (15), (Fig. 4a, b) were identified in extracts of *S. cerevisiae* expressing the genes encoding *Tw*CYP82D274 and *Tw*CYP71BE86. Compounds 9-12 all lack the C-3(C-4) double bond which could possibly cause stereochemical constraints of the C-3 and C-4 methyl’s preventing lactone formation (Fig. 4a). Assuming that the 18(4→3) abeo-abietane methyl shift is facilitated by *Tw*CYP71BE86 catalyzed formation of a carbocation, these compounds could be considered shunt products from an early quenching of the carbocation leading to a failure of establishing the C-3(C-4) double bond and the lactone (Fig. 4b). In this scenario *Tw*CYP71BE86 would go through multiple catalytic cycles, by initially catalyzing formation of the carbocation of C-3 causing the 18(4→3) abeo-abietane methyl shift and subsequent oxygenations of C-18 resulting in the formation of 6, and possibly also 9, 10, 11, and 12. If *Tw*CYP71BE86 utilizes a hypothetical Wagner-Meerwein mediated C18(4→3) rearrangement reaction (W.-M.) to account for the methyl shift of 1-8 to C-3 in the abietane carbon backbone, Cpd I and Cpd II represents states of the heme in the CYP catalytic cycle. Red line at heme bound hydroxyl symbolizes inhibition of oxygen rebound facilitating e- transfer as proposed by43 for CYP mediated carbocation formation. Compound 13 and oxygenated compounds hereof including 14, 17, and 18, would be products caused by the failure to inhibit oxygen rebound and additional oxygenation likely to be catalyzed by the *Tw*CYPs heterologously expressed in *S. cerevisiae*. Alternatively, the C18(4→3) could be mediated via a mechanism relying on the radical formed by the Compound II (CpdII) state of the involved CYP enzyme (Supplementary Fig. 7).

![Possible biosynthetic routes](image1)

![Hypothetical Wagner-Meerwein mediated C18(4→3)](image2)

(Fig. 4 | Proposed biosynthetic pathway of triptonide (2) from 14-hydroxy-dehydroabietadiene (5) in *S. cerevisiae*. a The biosynthetic pathway from 5 to 2 illustrating the formation of oxygenated miltiradiene derived intermediates catalyzed by the action of *Tw*CYP82’s (blue arrows) and *Tw*CYP71BE’s (red arrows) on the A- and C-ring of the abietane diterpene backbone, respectively. Underlined compounds or diastereomers hereof have previously been identified in plant extracts from *Tripterygium* species (Supplementary Table 1). b A hypothetical Wagner-Meerwein rearrangement reaction (W.-M.) to account for the methyl shift of C-18 to C-3 in the abietane carbon backbone. Cpd I and Cpd II represents states of the heme in the CYP catalytic cycle. Red line at heme bound hydroxyl symbolizes inhibition of oxygen rebound facilitating e- transfer as proposed by43 for CYP mediated carbocation formation. Compound 13 and oxygenated compounds hereof including 14, 17, and 18, would be products caused by the failure to inhibit oxygen rebound and additional oxygenation likely to be catalyzed by the *Tw*CYPs heterologously expressed in *S. cerevisiae*. Alternatively, the C18(4→3) could be mediated via a mechanism relying on the radical formed by the Compound II (CpdII) state of the involved CYP enzyme (Supplementary Fig. 7).
mechanism similar to CYP16IC241, failure to inhibit the O-rebound at the TuCYP71BE86 active site could explain the formation of 13, that subsequently is oxygenated causing the accumulation of 14, 17, and 18 (Fig. 4a).

Cationic rearrangements facilitated by CYP catalysis have previously been suggested44 and substantial experimental support has been provided to show that CYPs can catalyze a Wagner–Meerwein shift. Nevertheless, it cannot be completely excluded that the C18(4→3) shift proceeds via a free radical formed at CpdII (Supplementary Fig. 7), or that both cationic and free radical based reactions are occurring in parallel. If so, this may represent an intriguing evolutionary strategy offering the opportunity to increase the structural diversification of natural products in the plant kingdom by the ability of some enzymes to convert a specific substrate into different products by using different types of catalytic reaction mechanisms.

Out of the five *T. wilfordii* cytochrome *b*5 enzymes tested, only Tuycyt*b*5-A stimulated the activity of TuCYP71BE86 in *S. cerevisiae*41. A similar cytochrome *b*5-mediated stimulation has been observed in artemisinic acid biosynthesis catalyzed by AaCYP71AV1. While data on appropriate POR/cyt*b*5 expression ratios for heterologous biosynthesis of plant diterpenoids in *S. cerevisiae* has not been reported45, we hypothesize that differences in expression ratios could explain the disparate effects of Tuycyt*b*5-A expression in *S. cerevisiae* and *N. benthamiana*. In cytochrome *b*5 titration studies with mammalian CYPs, CYP activity was inhibited at specific ratios46. Alternatively, or in addition, differences in ER membrane properties, native POR and cytochrome *b*5 enzymes, or unknown factors in the two distinct hosts used for heterologous triptolide biosynthesis could influence Tuycyt*b*5-A functionality. The different effects of Tuycyt*b*5 co-expression show the advantage of using orthogonal systems for in vivo characterization of biosynthetic genes involved in plant specialized metabolism.

The TuCYP71BE86 associated formation of 6 and 7 requires a multiplicity of consecutive catalytic cycles. To facilitate these, electrons from TuPOR and/or Tuycyt*b*5-A need to be readily available. Presence of CYP reducing partners oxidizing NADPH and NADH, respectively, could promote the availability of electrons for an orchestrated series of CYP oxidations. Failure to complete these concerted oxidations could account for the accumulation of a number of the diterpenoid products formed in the *S. cerevisiae* triptolide biosynthesis strain.

In this study we demonstrate that biosynthesis of 2 from 3 can be catalyzed by CYPs from the CYP71BE and CYP82D subfamilies involving unique reactions including a methyl shift on the A-ring and multiple epoxidations of the B/C-ring system, respectively. Epoxides are generally highly reactive and typically labile moieties. LC-qTOF-MS analysis of extracts and NMR analysis of purified diterpenoids from *S. cerevisiae* afforded 2 as the only epoxide carrying compound identified. A triepoxide with a conformation of epoxide rings identical to 2 is the only epoxide containing diterpenoid observed in *T. wilfordii* tissue cultures47. Thus, derivatives of 3 with one or two epoxide rings have neither been identified in the native plant nor in the heterologous expression hosts described here. Instead, two abietane quinones were identified, whereof 18 and 16 have previously been isolated from *Tripterygium hongkouiana* and *T. wilfordii*48,49. To achieve efficient formation of the unique triepoxide configuration, TuCYP82D274 and TuCYP82D2713 could be required to pass through multiple highly coordinated consecutive catalytic cycles, possibly facilitated by metabolon formation18. Intermediates or products escaping the concerted oxidations including diterpenoids with one or two epoxide rings are possibly less stable compounds that rearrange into quinone diterpenoids. Further studies including evaluation of the stability of mono- and dioepoxide abeo-abietane diterpenoids possibly derived by chemical synthesis would be required to clarify some of these questions and hypotheses.

In addition to being organized in metabolons, orchestration of plant biosynthetic pathways may also be controlled at the genome level e.g. by localization of the biosynthetic genes in gene clusters46. The enzymes identified here, including close orthologs of TuCYP82D274 and TuCYP71BE86, were found in tandem repeats on the chromosomes CM023857, CM023867, and CM023888 (Fig. 1c and Supplementary Table 23). These tandem repeats could have emerged from gene duplication throughout the evolution of this plant species50. Multiple copies of these genes could enable *T. wilfordii* to achieve high levels of the CYP enzymes via simultaneous transcription from multiple biosynthetic genes in the genome. With the diTPS genes encoding the first committed step in biosynthesis of 2 being co-localized on the same chromosome as TuCYP71BE86/88, the biosynthetic pathway of 2 in *T. wilfordii* could be considered clustered51. Still, while TuCYP71BE86 can accept 3 as substrate (Supplementary Fig. 3) in planta, it remains to be determined how the derived products contribute to the biosynthesis of 2 or whether miltiradiene derived products from TuCYP82D274 catalysis are more appropriate substrates. Thus, it is unclear how and in which order the TuCYP71BE86 and TuCYP82D274 contribute to the biosynthetic step(s) following the formation of 3.

Considering that co-expression of four CYPs in two orthologous host systems resulted in the formation of several miltiradiene derived compounds also observed in *Tripterygium* species, a linear pathway might be considered a too simplistic model for the biosynthesis of 2 in *T. wilfordii*. Instead, broad substrate and product promiscuity of the TuCYPs could suggest that 2 is only one out of many miltiradiene derived products in *Tripterygium* species that emerges from a grid-type biosynthetic pathway with involvement of a limited number of biosynthetic enzymes (Fig. 4a).

To conclude, the identification of CYPs catalyzing unprecedented types of reactions in plant diterpenoid biosynthesis significantly advances our understanding about their diverse catalytic capacities. This finding will guide future efforts in assigning CYPs to biosynthetic pathways with reaction steps not easily explained as classical CYP catalyzed reactions. Diterpenoid scaffold diversity have until now mainly been associated with the catalytic capacity of diTPS while CYPs have been associated with further decoration of the core structures by monoxygenation reactions48. Our data demonstrate that plant CYPs may also play a key role in the modification of the basic diterpenoid core structures. In the case of the biosynthesis of 2, these unique CYPs are harbored within the CYP71BE subfamily possibly catalyzing a Wagner-Meerwein reaction resulting in scaffold diversification of labdane diterpenoids, including formation of the 18(4→3) abeo-abietane backbone essential for biosynthesis of 2.

By introducing the genes shown to be involved in formation of 2 into yeast, we have established proof-of-concept for an on-demand and scalable production platform for 2 as a replacement for the current plant extraction-based production of this high value triepoxide diterpenoid.

**Methods**

**Establishing constructs for high yield miltiradiene biosynthesis in N. benthamiana**

For enhanced biosynthesis of miltiradiene, and to employ a gene assembly system that was compatible with the EasyCloni system43, we designed a new plasmid system for transient expression in *N. benthamiana*. Promoters for the new vector were sourced from literature and obtained via DNA synthesis (pCM9, pSGT54,55), from Addgene (pSIM24 – pM24 promoter) or from in-house DNA template (p35S - pLIFE33). A synthetic DNA Gblock was ordered from Integrated DNA Technologies (IDT, USA) containing the terminator tOCS18 and t3A terminator connected by the USER cassette 5′-CAACGGATGCCTGCAGCTCGTGAATTC-3′. The new...
**N. benthamiana** expression vector was established by introducing the TOCS·USER cassette·t3A construct into the pLIFE33 backbone amplified with primer pairs GB3A31 + GB3A32, by InFusion cloning (Takara Bio, USA). Assembled plasmid was transformed into E. coli 10 G cells (Lucigen, USA). Plasmid sequence was verified by Sanger sequencing (Macrogen, South Korea) and named New_pLIFE. Genes selected for transient expression in N. benthamiana were cloned into the established plasmids by using methods described in the Easy-Cloni system. While working on the data presented here, it was shown that transient co-expression of GGPPS and dTPPS encoding genes targeted to the cytosol, together with HMGR in *N. benthamiana* provides enhanced diterpenoid accumulation. Employing a similar strategy, we constructed two dual expression constructs by cloning SchHMGR, together with a bidirectional promoter and SpGGPPS in the New_pLIFE plasmid. On another New_pLIFE plasmid TcCYP71A, was assembled with a bidirectional promoter and TcCYP71B. The bidirectional promoter consisted of pM24 and Cm9. Combined the four genes in the two expression construct are denoted the miltiradiene biosynthetic genes.

**Isolation of *T. wilfordii* CYP genes and expression in *Nicotiana benthamiana***

Tripterygium wilfordii CYP genes (TuCYPs) were cloned from cDNA synthesized using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher) from RNA isolated from either root, stem or leaf material from *T. wilfordii*. Genes were cloned into pLIFE33 or New_pLIFE by USER cloning. A complete list of tested TuCYPs is provided in Supplementary Table 21.

Full length TuCYP genes were transiently co-expressed with the miltiradiene biosynthetic genes in *N. benthamiana* using agrobacterium mediated transfection. Briefly, binary vectors each containing the miltiradiene biosynthetic genes or TuCYPs were transformed by electroporation into agrobacterium. Liquid cultures of transformed agrobacterium each containing specific plasmids were mixed for co-expression. Leaf material of *N. benthamiana* co-expressing specific combinations of TuCYPs together with the miltiradiene biosynthetic genes was harvested 7 d after agrobacterial infiltration. 1 mL MeOH was added to two leaf disks (~2 cm). Extraction was done at room temperature at 200 rpm orbital shaking. 200 µL of extract were filtered by using a 0.22 µm 96-well filter plate (Merck Millipore, Darmstadt, Germany) and at stored at 4 °C prior to LC-MS analysis.

**Saccharomyces cerevisiae growth media**

YPD media: 20 g/L Bacto™ Peptone, 10 g/L Bacto™ Yeast extract, 20 g/L glucose.

Synthetic complete (SC) media without uracil: 1.92 g/L Yeast Extract, 10 g/L Bacto™ Peptone, 20 g/L glucose.

**Extraction and metabolite analysis**

Genetically engineered *S. cerevisiae* strain was transferred into 0.5 mL media in a 96-well plate and grown for 3 d at 30 °C with orbital shaking at 350 rpm. For extraction, 0.1 mL of *S. cerevisiae* culture was transferred to 1.5 mL glass vials. 0.4 mL MeOH uHPLC grade was added. *S. cerevisiae* extracts were filtered by using a 0.22 µm 96-well filter plate (Merck Millipore, Darmstadt, Germany) and at stored at 4 °C prior to LC-MS analysis.

**LC-MS analysis**

MeOH extracts were analysed using an Ultimate 3000 UHPLC + Focused system (Dionex Corporation, Sunnyvale, CA) coupled to a Bruker Compact ESI-QTOF-MS (Bruker) system. Samples were separated on a Kinetex XB-C18 column (100 × 2.1 mm ID, 1.7 µm particle size, 100 Å pore size; Phenomenex Inc., Torrance, CA) maintained at 4 °C prior to LC-MS analysis. Three LC protocols were used:

1. LC method 1: 0–0.5 min, 10 % B; 0.5–21 min, linear increase from 10 to 80 % B; 21–31 min, to 90 % B; 31–34 min, to 100 % B; 34–39 min 100 % B; 39–40 min linear decrease from 100 to 10 % B. Isocratic 20 % B 41–45.5 min.
2. LC method 2: 0–0.5 min, 20 % B; 0.5–11 min, linear increase from 20 to 80 % B; 11–20 min, 100 % B; 20–22 min, 100 % B; 22–27 min linear decrease from 100 to 20 % B. Isocratic 20 % B 28–32 min.
3. LC method 3: 0–0.5 min, 20 % B; 0.5–9 min, linear increase from 20 to 100 % B; 9–11 min, 100 % B; 11–15 min, linear decrease from 100 to 20 % B; 15–15 min, 20 % B.
Mass spectra were acquired in positive ion mode over a scan range of m/z 50–1200 with the following ESI and MS settings: capillary voltage, 4000 V; end plate offset, 500 V; dry gas temperature, 220 °C; dry gas flow of 8 L/min; nebulizer pressure, 2 bar; in source CID energy, 0 eV; hexapole RF, 50 Vpp; quadrupole ion energy, 4 eV; collision cell energy, 7 eV. Raw chromatogram data was calibrated using an internal sodium formate standard and subsequently exported as *mzML format using DataAnalysis 4.3 (Bruker). MZmine ver 2.53 was used for visualizing the LC-MS chromatograms.

GC-MS analysis
GC-MS analysis was carried out on a Shimadzu GCMS-QP2010 Ultra (Shimadzu Corp.) with an Agilent HP-5MS column (Agilent Technologies) 20 m × 0.18 mm i.d., 0.18 µm film thickness). Hydrogen was used as a carrier gas at a constant linear velocity of 50 cm s⁻¹, and the injection volume was 1 µL at 250 °C (splitless mode). The oven program was 80 °C for 2 min, ramp at rate 20 °C/min to 180 °C, ramp at rate 10 °C/min to 300 °C, ramp at rate 20 °C/min to 310 °C, hold for 3 min. Data was stored in.CDF format and processed in MZmine2.

Relative quantification of mitrradiene derived diterpenoids
Relative compound quantities in yeast cultures were based on normalized peak areas of characteristic ions (data obtained using targeted feature detection in the MZmine2 software). The signal for the following ions were quantified: 3: m/z 255 (GC-MS), 5: m/z 189 (GC-MS), 13: m/z 303.2316, 6: m/z 283.2039, 7: m/z 299.2002, 8: m/z 313.1794, 2: m/z 359.1481. For LC-qTOF-MS and GC-MS data a mass deviation of 5 ppm and 100 ppm, respectively, was tolerated.

For LC-qTOF-MS, the peak area of the base peak ion (m/z 315.1947) for the internal standard andrographolide was used for normalization. Additional data analysis of normalized peak areas were done with Microsoft Excel for Mac. Version 16.59 (Microsoft Inc.) and SigmaPlot Version 14.5 (Systat Software Inc.).

Absolute quantifications of 2 and 6 from feed-batch fermentation
Absolute quantifications of 8 (FT65732, CarboSynth) and 2 (FT65197, CarboSynth) were carried out by co-analysis of authentic standards prepared in MeOH and a final concentration of 5 ppm internal standard (andrographolide). Quantification was based on normalized peak area and calculated from the slopes of linear extrapolations of the standard response curve (triphotenolide 0.05, 0.5, 1, 2 ppm; triptonide 0.5, 1, 2, 10, 20 ppm).

Metabolomics, identification of peaks observed in N. benthamiana and S. cerevisiae expressing the triptonide biosynthetic genes
LC-MS data from duplicate samples of the negative control and triptonide biosynthesis samples were analyzed using MZmine ver2.53.a Briefly, noise level was set to 1.5E4, ADAP Chromatogram builder was used for feature detection, while chromatographic peaks were detected by Chromatogram deconvolution using the local minimum search algorithm. Monoisotopic masses were identified using the Isotope peak grouper with a tolerance of m/z 0.01 or 10 ppm, while peaks in the N. benthamiana and S. cerevisiae extract samples, respectively, were aligned using a m/z and retention time tolerance of 5 ppm and 0.15 min. All peaks not identified in both of the duplicate samples were removed. Peaks exclusively identified the triptonide biosynthesis samples while not appearing in the background samples were retained. Identification of oxygenated diterpenoids or conjugates thereof was based on the molecular formula predicted from the accurate mass of monoisotopic peaks (<5 ppm). Putative monoisotopic peaks resulting from in source fragmentation of diterpenoid conjugates were removed.

Feed-batch fermentation of engineering S. cerevisiae strains for isolation of mitrradiene derived diterpenoids
All engineered S. cerevisiae strains were cultivated in 96-deepwell plates using a Feed-In-Time (FIT; m2p-labs)²⁰. For isolation and purification of key intermediates in the triptonide pathway selected engineered S. cerevisiae strains were cultivated in feed batch fermentor using a 2 L Biostat® A bioreactor (Sartorius AG).

Batch media contained: 55 g/L glucose − H2O, 25 g/L (NH4)2SO4, 1.7 g/L MgSO4 − 7H2O, while feed media consisted of: 880 g/L glucose − H2O, 21.6 g/L KH2PO4, 24.24 g/L MgSO4 − 7H2O, 8.4 g/L K2SO4, and 0.672 g/L NaNH2SO4. Batch- and feed salt mixes as well as batch and feed glucose were prepared separately by dissolving components in Milli-Q water and sterilizing by autoclaving. The feeding solution was made by mixing 500 mL of feed glucose with 500 mL of feed salt mix, 10 mL of vitamin mix (0.64 g/L D-biotin, 3 g/L nicotinic acid, 10 g/L thiamin HCl, 4 g/L D-pantothenic acid hemicalcium salt, 8 g/L myo-inositol, 2 g/L pyridoxine HCl), 10 mL microelements (6.7 g/L Titrplex III, 6.7 g/L (NH4)2Fe(SO4)2 · 6H2O, 0.55 g/L CuSO4 · 5H2O, 2 g/L ZnSO4 · 7H2O, and MnSO4 · H2O), and 1 mL of trace elements solution (1.25 g/L NiSO4 · 6H2O, 1.25 g/L CoCl2 · 6H2O, 1.25 g/L KI, and 1.25 g/L Na2MoO4 · 2H2O).

Fed batch fermentation was initiated by addition of a 100 mL standards culture to the reactor tank (with impellers), which in turn was prepared earlier by autoclaving while containing 200 mL Batch glucose and 300 mL Batch salt mix. Also 5 mL vitamin mix, 5 mL micro elements and 0.5 mL trace elements, were added. Cultivation in the bioreactor was started under the following conditions (monitored and automatically controlled): pH = 5, temperature = 30 °C, dissolved oxygen (DO) = 20%. While pH was controlled by feeding of ammonium hydroxide (32%) and sulfuric acid (10 %), dissolved oxygen was controlled by air supply combined with stirring. Also foam levels were adjusted by addition of anti-foam emulsion (3519, Serva Electrophoresis GmbH). After 18 h of initial cultivation in the bioreactor, feeding with Feeding solution at a rate of 1.3% was started. The fermentation process continued for 7 d with daily sampling of the culture.

Isolation and purification of mitrradiene derived compounds from engineered S. cerevisiae strain for NMR analysis
Compounds in this invention were isolated from bioreactor cultured yeast strains NVJ8.15, and NVJ5.10, and structurally elucidated by NMR. The combined ethyl acetate extracts of broth and MeOH-lysed cells (cells/MeOH = 1:4, v/v) were initially dried in presence of Celite S* (06858, Sigma-Aldrich) via rotary evaporation. Compounds were subsequently isolated by successive fractionations using a puriFlash® 5.250 (Interchim, Montluçon, France) instrument with detection by UV absorbance and Evaporative Light-Scattering Detection (ELSD). This was equipped with a s PF-3S1HP-F0025 (CI) column (OV002A, Interchim) for normal phase and a US5C15HQQ-100/300 (C2) column (SSP750, Interchim) - for reverse phase separation.

An initial pre-fractionation of the dry mix of Celite S*/crude extract was achieved using column (CI) with loading from a manually packed dry-loading column. Separation was obtained using mobile phases hexane (A) and ethyl acetate (B), a constant flow rate of 15 mL/min, followed by a final washing step with 100% MeOH. Compounds of interest were detected by UV and ELSD and collected. Collected fractions were continuously evaluated by LCMS using LC-MS method 3 and TLC analysis prior to further fractionation or NMR studies. Additional purification of compounds of interest from fractions containing multiple compounds was carried out by an additional normal phase fractionation using CI1 or a reverse phase column fractionation using CI2.

For reverse phase purification with C2, sample solvents were evaporated using rotor evaporation and the residue resuspended in 2 mL MeOH. Sample was injected directly onto the pre conditioned column C2. Mobile phases for C2 consisted of solvent C: deionized
water and solvent D acetonitrile each acidified with 0.05% (v/v) formic acid. A constant flow rate of 32 mL/min was used, with a linear solvent gradient with increasing concentration of solvent D. Compounds of interest were detected by ELSD and UV and collected.

Additional reverse phase purification was done by multiple injections of 100 μL onto a semi-prep Phenomenex Luna 5 μm C18(2) 100 Å 250 × 10 mm. (Fully porous) (Phenomenex, Inc., Torrance, CA, USA) column on a Shimadzu HPLC (SPD-M20A diode array detector, FRC-10A fraction collector, DGU-20A degasser, LC-20AT pump, CBM-10A System controller, CTO-10AS VP column oven, SIL-10AP autosampler). Mobile phase was a linear gradient between C and D with an increasing amount of D going from 50–100%. Compounds of interest were detected by UV absorbance at 210 nm and collected.

NMR analysis
NMR experiments were acquired on a Bruker Avance III HD 600 MHz NMR spectrometer (H operating frequency 599.85 MHz) equipped with a 5-mm cryogenically cooled DCH cryoprobe optimized for 13C and 1H or a Avance III 600 MHz spectrometer (H operating frequency 600.13 MHz) equipped with a 1.7-mm TCI cryoprobe (Bruker Biospin, Karlsruhe, Germany). NMR data was recorded in 1.7- or 5-mm tubes in CDC13 or CD3OD (Euriso-top, 99.8 atom % D) with temperature equilibration to 300 K, optimization of lock parameters, gradient shimming, and setting of receiver gain, all automatically controlled by Topspin ver. 3.2 or 3.5 and IconNMR ver. 4.7.5 or 5.0.7 (Bruker Biospin, Karlsruhe, Germany). 1H and 13C chemical shifts were referenced to the residual solvent signals at respectively δH 7.26 ppm and δC 77.16 ppm (CDCl3) and δH 3.31 ppm and δC 49.00 ppm (CD3OD). 1D and 2D NMR spectra were acquired with 30° pulses and 64k data points and 1H or a Avance III 600 MHz spectrometer (1H operating frequency (CDCl3) and δC 49.00 ppm (CD3OD). 1D and 2D NMR data was acquired with 4096 (HMBC), 2048 (DQF-COSY and ROESY), or 1024 (multiplicity edited HSQC) data points in the direct dimension and 256 (DQF-COSY, HMBC and ROESY) or 256/128 (multiplicity edited HSQC) data points in the indirect dimension. 2D NMR data was zero−decoupled using the Waltz-16 composite pulse decoupling scheme. 2D homo- and heteronuclear experiments were acquired with 4096 (HMBC), 2048 (DQF-COSY and ROESY), or 1024 (multiplicity edited HSQC) data points in the direct dimension and 256 (DQF-COSY, HMBC and ROESY) or 256/128 (multiplicity edited HSQC) data points in the indirect dimension. 2D NMR data was zero−decoupled to 1H in F1 and zero−decoupled to twice the number of points in F2, employing forward linear prediction in F1 (LPBIN = 0). The 2D experiments supported the results from the 1D-experiments and structures of the identified molecules in Supplementary Figs. 8–33 and Supplementary Table 1–19. Processing of NMR data was done using Topspin ver. 4.0.9 (Bruker Biospin, Karlsruhe, Germany).

Optical rotation was conducted on a ADP410 from Bellingham and Stanley, using MeOH as a reference. Column length was 20 cm and pure compound was dissolved in 10 mL of MeOH. See Supplementary Note 4 for optical rotation measurement values and the comments.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Sequence information data for TwCYP71BES5 (accession ON375998), TwCYP71BE68 (accession ON375999), TwCYP82B213 (accession ON376000), TwCYP82B274 (accession ON376001), Twb5-A (accession ON376002), Twb5-B (accession ON376003), Twb5-C (accession ON376004), Twb5-D (accession ON376005), Twb5-E (accession ON376006), Twb5-F (accession ON376007), and TwPOR1 (accession ON376008) are available in the NCBI GenBank. Source data are provided with this paper.

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Acknowledgements
The authors would like to thank Fernando Geu-Flores for his input on the hypothesized mechanisms for the CYP catalyzed C18(C4→C3). This research has been funded by grants from the Novo Nordisk foundation (NNF18OC0031974 [J.A.R.], NNF20OC0061048 [J.A.R.], and NNF16OC0021616 [B.L.M, D.S.]), The Danish Innovation Fund (0160-000168) [J.A.R] and the Lundbeck foundation (R223-2016-85 [B.L.M., D.S.]).

Author contributions
N.L.H, B.L.M., and J.A.R. conceived and initiated the study. N.L.H., and J.A.R. Identified, and isolated genes from plant cDNA, performed N. benthamiana expression studies, metabolite analysis and isolation of compounds for NMR analysis. N.L.H., V.F., Q.H. and J.A.R. established yeast strains used. L.K. and D.S. conducted the NMR analysis. B.L.M. and J.A.R. wrote the manuscript. N.L.H., V.F., Q.H., D.S., L.K., B.L.M. and J.A.R. revised the manuscript.

Competing interests
N.L.H., V.F., and J.A-R. are inventors of the patent entitled “Production of oxygenated diterpenoid compounds” (application number: PCT/EP2021/073656) related to the cytochrome P450 enzymes described in the paper. J.A-R. has established TriptoBIO to commercialize the patented technology. Other authors claim no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-32667-5.

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Peer review information Nature Communications thanks Shengying Li and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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