Identification of a cytochrome P450 from *Tripterygium hypoglaucum* (Levl.) Hutch that catalyzes polpunonic acid formation in celastrol biosynthesis

CHEN Xiao-Chao\(^1\), LU Yun\(^1\), LIU Yuan\(^1\), ZHOU Jia-Wei\(^1\), ZHANG Yi-Feng\(^2\), GAO Hai-Yun\(^1\), LI Dan\(^1\), GAO Wei\(^1,2\)*

\(^1\) School of Traditional Chinese Medicine, Capital Medical University, Beijing 100069, China; \(^2\) Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China

Available online 20 Sep., 2022

**[ABSTRACT]** *Tripterygium hypoglaucum* (Levl.) Hutch, a traditional Chinese medicinal herb with a long history of use, is widely distributed in China. One of its main active components, celastrol, has great potential to be developed into anti-cancer and anti-obesity drugs. Although it exhibits strong pharmacological activities, there is a lack of sustainable sources of celastrol and its derivatives, making it crucial to develop novel sources of these drugs through synthetic biology. The key step in the biosynthesis of celastrol is considered to be the cyclization of 2,3-oxidosqualene into friedelin under the catalysis of 2,3-oxidosqualene cyclases. Friedelin was speculated to be oxidized into celastrol by cytochrome P450 oxidases (CYP450s). Here, we reported a cytochrome P450 *ThCYP712K1* from *Tripterygium hypoglaucum* (Levl.) Hutch that catalyzed the oxidation of friedelin into polpunonic acid when heterologously expressed in yeast. Through substrate supplementation and *in vitro* enzyme analysis, *ThCYP712K1* was further proven to catalyze the oxidation of friedelin at the C-29 position to produce polpunonic acid, which is considered a vital step in the biosynthesis of celastrol, and will lay a foundation for further analysis of its biosynthetic pathway.

**[KEY WORDS]** *Tripterygium hypoglaucum*; Cytochrome P450; Polpunonic acid; Celastrol; Biosynthesis

**[CLC Number]** R284

**[Document code]** A

**[Article ID]** 2095-6975(2022)09-0691-10

---

**Introduction**

*Tripterygium hypoglaucum* (Levl.) Hutch, a woody vine from the family Celastraceae native to western and southern China, is a traditional Chinese medicinal herb that was first recorded in *Compendium of Materia Medica* (Bencao Gangmu) \(^{[1,2]}\). It has been used for the treatment of rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and chronic urticaria \(^{[3-4]}\). Modern pharmacological studies indicated that *T. hypoglaucum* exhibited strong anti-inflammatory and immunosuppressive effects \(^{[5,7]}\), which are mainly derived from a variety of compounds accumulated in the roots, including active alkaloids, diterpenoids and triterpenoids \(^{[7]}\).

Celastrol is a red methylated quinone triterpenoid that is mainly present in the root bark of certain plants in the family Celastraceae \(^{[7]}\). In 1936, it was isolated from the roots of *Tripterygium wilfordii* for the first time \(^{[8]}\), and then from a variety of plants in the Celastraceae family afterwards, such as *Celastrus orbiculatus* Thunb. and *T. hypoglaucum*. In recent years, increasing attention has been drawn on celastrol due to its great medicinal and commercial value. Celastrol exhibits strong effects against several human diseases, with a potential role in the treatment of chronic inflammatory disorders, such as arthritis \(^{[9]}\), lupus erythematosus \(^{[10]}\), lateral sclerosis \(^{[11]}\), and Alzheimer’s disease \(^{[12]}\). It also has the potential to prevent obesity and metabolic dysfunction through activating the transcription factor HSF1 and to inhibit angiogenesis-mediated tumor growth \(^{[13]}\). However, there are practical reasons that limit its further application, such as the slow growth rate, yield fluctuations and restricted planting area of the celastrol producing plants. Although there are reports concerning the
total synthesis of celastrol, it has not been applied on a preparative scale due to the complicated and inefficient synthesis process. As an alternative, heterologous recombinant microbial biosynthesis pathways may be an effective solution for the sustainable and reliable supply of celastrol.

Celastrol is a triterpene compound of the friedelane type. The proposed biosynthetic pathway shown in Fig. 1 illustrates some of the biosynthetic steps of celastrol. Friedelin is derived from the secondary metabolites of plants and shares the upstream biosynthetic pathway with other triterpenes and sterols. Most triterpenes and sterols are synthesized from 2,3-oxidosqualene, which is derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) derived from the mevalonate (MVA) pathway and methylerythritol phosphate (MEP) pathway, respectively. Subsequently, two molecules of IPP and one molecule of DMAPP are condensed to form farnesyl diphosphate (FPP) by a specific synthase. Squalene synthase (SQS) converts two molecules of FPP into squalene, and then squalene epoxidase (SQE) oxidizes squalene into 2,3-oxidosqualene, the common precursor of triterpenes and sterols. Oxidosqualene cyclase (OSC) catalyzes the complicated cyclisation of 2,3-oxidosqualene to form friedelin, which is considered the first step in the formation of the friedelin skeleton. Zhou et al. proposed a potential synthetic pathway based on reported metabolomic data. Friedelin is the precursor of celastrol and the subsequent steps are considered to be further catalyzed by CYP450s that form polpunonic acid, wilforic acid C and other compounds. These intermediates undergo a series of oxidation steps to form celastrol. It has been reported that CYP450s of CYP712K family from only two species can convert friedelin into polpunonic acid, including TwCYP712K1, TwCYP712K2, TwCYP712K3 and McCYP712K4. These findings provide useful genetic resources for further studies and lay a foundation for exploring the biosynthetic pathway of celastrol.

In this work, ten CYP450 genes were screened from the transcriptome data of *T. hypoglucum* based on tissue-specific expression patterns. After full-length cloning, seven CYP450 genes were obtained and their functions were characterized through heterologous expression in yeast. One of these CYP450s was found to exhibit catalytic activity towards the target substrate and named *ThCYP712K1*. Furthermore, it catalyzed the oxidation of friedelin at the C-29 position to generate polpunonic acid in vitro and in vivo. This finding may be of great significance for promoting the study of this species at the molecular level and finally achieving the commercially viable heterologous biosynthesis of celastrol and other friedelin-derived compounds.

**Materials and Methods**

**Plant materials**

Nature plant tissues of *T. hypoglucum* were collected from Fujian Province in 2019 and identified by TU Yu-He, a...
teacher from the State-owned Forest Farm. Samples of roots, stems and leaves were harvested from three individual plants frozen in liquid nitrogen and stored at −80 °C.

**RNA isolation and cDNA synthesis**

Total RNA of *T. hypoglauca*um plant tissues (including roots, stems, and leaves) was extracted using a modified cetyltrimethylammonium bromide (CTAB) method [25]. The total extracted RNA was qualified by 1.5% agarose gel electrophoresis, before purification using an RNA Purification Kit (Tiangen Biotech, Beijing, China). After the concentration was determined, it was directly used for the synthesis of cDNA. First-strand cDNA was reversely transcribed from the purified RNA using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, USA), according to the manufacturer’s instructions.

**Gene discovery and expression analysis**

Transcriptome data of *T. hypoglauca*um were used to screen candidate CYP450 sequences based on functional annotation results. The gene expression heat map was plotted using TBTools software (China), which showed the distribution of candidate genes in different tissues. All the cloned candidate genes were named by the Nomenclature Committee (David Nelson: dnelson@uthsc.edu).

**Phylogenetic analysis**

Amino acid sequences of CYP450s from *T. hypoglauca*um were obtained using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequences of CYP450s from other plants were downloaded from the NCBI database. Sequence alignments were conducted in MEGA7 using MUSCLE. A neighbor-joining tree was built using MEGA7 via the bootstrap method with 1000 replications.

**Gene cloning of candidate CYP450s**

The full-length open reading frames (ORFs) were cloned by polymerase chain reaction (PCR) using Phusion High-Fidelity PCR Master Mix (New England BioLabs, USA) according to the manufacturer’s instruction. All the primers are listed in Supplementary Table S2. The PCR conditions are listed as follows: initial denaturation at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 60 °C for 15 s and 72 °C for 90 s, and final extension at 72 °C for 5 min. The PCR products were ligated into the pEASY®-Blunt zero cloning vector (TransGen Biotech, Beijing, China), and the recombinant vector was used to transformed *E. coli* Trans-T1 cells. Transformants were selected on Luria-Bertani (LB) agar plates with 100 mg L⁻¹ ampicillin (AMP) at 37 °C overnight. The positive colonies were verified by bacterial PCR and sequencing.

**Functional characterization in yeast in vivo**

**Construction of the yeast expression vector**

The ORF of *TwCPR3* was amplified and introduced into the yeast epitope tagging vector pESC-Leu under the control of the inducible GAL1 promoter. Then, *TwOSC1* was amplified and subcloned into the same vector under the control of the inducible GAL10 promoter according to the protocol of the pEASY®-Uni Seamless Cloning and Assembly Kit (TransGen Biotech). The ORF sequences of CYP450s were amplified and subcloned into the pYES2 expression vector (Invitrogen) using the same method.

**Heterologous expression of candidate genes in yeast**

The pYES2 vector containing a CYP450 candidate gene and recombinant plasmids containing *TwOSC1* and *TwCPR3* were introduced into lanosterol synthase-deficient yeast (*Saccharomyces cerevisiae*, purchased from ATCC, cell line number: 4 021 900, -ERG7, -Ura). All primers used in this section are shown in Supplementary Table S3. The transformants were selected on solid synthetic complete medium without uracil and leucine (SC-Ura-Leu) for selection. The positive transformants were cultured in 5 mL SC-Ura-Leu liquid medium containing 2% glucose in a 15 mL flask and incubated at 30 °C at 200 r·min⁻¹ for 24 h. Subsequently, the yeast cells were transferred to a 100 mL shake flask containing 30 mL of SC-Ura-Leu liquid medium with 2% glucose and incubated at 30 °C at 200 r·min⁻¹ for 8−12 h. Thereafter, the yeast cells were centrifuged and resuspended in 30 mL of liquid medium containing yeast extract, peptone and 2% galactose and further induced at 200 r·min⁻¹ and 30 °C for 48 h to induce heterologous gene expression. Then the yeast cells were harvested by centrifugation (4 min, 4000 × g), and subjected to ultrasonic extraction with 20 mL ethyl acetate three times for 30 min each. All the extracts were combined and concentrated by rotary evaporation. The residue was reconstituted with ethyl acetate dried under an N₂ stream and then dissolved in methanol. The samples were analyzed by ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC/Q-TOF-MS) as listed in the Supplementary Table S4. The UPLC separation was performed using a Waters Acquity UPLC TM I-Class system (Waters Corp., Milford, MA, USA) with an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, USA) and samples were scanned in the absorption spectrum from 190 to 450 nm. The TOF MS analysis was performed using a Xevo G2-S QTOF MS system (Waters Corp., Milford, MA, USA). The positive ion mode was employed to ionize the chemical compounds. The detection conditions are listed as follows: capillary voltage at 1.0 kV, cone voltage 40 V, source temperature 120 °C, desolvation temperature 450 °C, cone gas flow 50 L·h⁻¹, and desolvation gas flow 800 L·h⁻¹. TOF-MS was set from 50 to 1500 m/ε. The scan time was 0.2 s. Sample data were collected and processed using MassLynx software.

**Substrate supplementation**

The ORF of the *TwCPR3* was amplified and introduced into the yeast epitope tagging vector pESC-Leu under the control of the inducible GAL1 promoter following the protocol of the pEASY®-Uni Seamless Cloning and Assembly Kit (TransGen Biotech). The plasmids containing *TwCPR3* were introduced into lanosterol synthase-deficient yeast (*Saccharomyces cerevisiae*, purchased from ATCC, cell line number: 4 021 900, -ERG7, -Ura) with each pYES2-Ura vector
containing a CYP450 candidate sequence. The primers used in this section are shown in Supplementary Table S3.

The transformants were selected on solid synthesis complete medium without uracil and leucine (SC-Ura-Leu). The positive transformants were cultured in 3 mL SC-Ura-Leu liquid medium containing 2% glucose in a 50 mL flask and incubated at 30 °C at 200 r·min⁻¹ for 24 h. Then, yeast cells were transferred to a 100 mL shake flask containing 30 mL SC-Ura-Leu liquid medium with 2% glucose and incubated at 30 °C at 200 r·min⁻¹ for 8 h. Thereafter, the yeast cells were pelleted and resuspended in 30 mL of liquid medium containing yeast extract, peptone and 2% galactose and further cultured at 200 r·min⁻¹, 30 °C for 12 h, to an OD₆₀₀ of 0.8–1. Friedelin and 29-hydroxy-friedelin-3-one were dissolved in DMSO to prepare a stock solution with a concentration of 3 mg·mL⁻¹. Friedelin and 29-hydroxy-friedelan-3-one were separately added to each flask to a final concentration of 30 μmol·L⁻¹. After fermentation for 48 h at 30 °C and 200 r·min⁻¹, the yeast cells were harvested by centrifugation (4,000 × g). The supernatant was discarded and the products were extracted for UPLC/Q-TOF-MS analysis as described above. In addition, the substrate spectrum was assessed by adding 1.5 mg·mL⁻¹ of lupeol, oleic acid, ursoic acid, betulinic acid, or α-amyrin, which have similar structure to friedelin, using the same assay conditions as described above.

**In vitro enzyme activity assays**

The in vitro enzyme activity assays were conducted as described before [26,27], with minor modifications. Yeast cells were harvested by centrifugation and resuspended in 50 mL bacterial resuspension containing 50 mmol·L⁻¹ Tris-HCl (pH 7.5), 1 mmol·L⁻¹ ethylenediaminetetraacetic acid (EDTA), 0.5 mmol·L⁻¹ phenylmethylsulfonyl fluoride (PMSF), 1 mmol·L⁻¹ dithiothreitol (DTT), 0.6 mol·L⁻¹ D-sorbitol and ddH₂O. High pressure cell disruption equipment (Constant Systems, Nortamptonshire UK) was used to lyse the yeast cells. After centrifugation, 42.5 mL of the supernatants were collected and supplemented with CaCl₂ to a final concentration of 18 mmol·L⁻¹. Microsomal proteins were then collected by centrifugation and suspended at a final protein concentration of 10 to 15 mg·mL⁻¹ in a storage buffer containing 50 mmol·L⁻¹ Tris-HCl (pH 7.5), 1 mmol·L⁻¹ EDTA and 20% (V/V) glycerol.

The catalytic activity of putative CYP enzymes was assayed in 500 μL reaction mixtures containing 1 mmol·L⁻¹ nicotinamide adenine dinucleotide phosphate (NADPH), 5 mmol·L⁻¹ flavin adenine dinucleotide (FAD), 5 mmol·L⁻¹ flavin mononucleotide (FMN), 5 mmol·L⁻¹ glucose-6-phosphate(G-6-P), 1 unit·mL⁻¹ glucose 6-phosphatasehydrogenase (G-6-P-D), 2 mmol·L⁻¹ DTT and 20 μg friedelin or 29-hydroxy-friedelan-3-one as substrate, and then the protein was added to make up the volume to 500 μL. After incubation at 30 °C, 100 r·min⁻¹ for 24 h, the reactions were extracted three times with an equal volume of ethyl acetate (AR, China). Pooled extracts were completely evaporated under N₂ and then residues dissolved in 200 μL of methanol for UPLC/Q-TOF-MS analysis.

**Comparison of catalytic properties between ThCYP712K1 and TwCYP712K1**

To compare the catalytic efficiency of isozymes, the pYES2 vector containing TwCYP712K1 or ThCYP712K1 were used together with TwOSC1 and TwCPR3 to co-transform the lanosterol synthase-deficient yeast as described before. The transformants were selected on SC-Ura-Leu solid medium and five positive colonies were selected and cultivated in 5 mL SC-Ura-Leu liquid medium containing 2% glucose in a 50 mL flask at 30 °C at 200 r·min⁻¹. Then, 30 mL of SC-Ura-Leu liquid medium with 0.2% glucose and 1.8% galactose was inoculated to an initial OD₆₀₀ of 0.1, and incubated at 30 °C at 200 r·min⁻¹ for 4 days. The products were extracted with the same methods as described before and then analyzed by Q Exactive HF (Thermo Scientific, Amercia). The conditions of liquid chromatography analysis are listed in Supplementary Table S5. The targeted single ion monitoring (SIM) mode was chosen to realize the relative quantification. The positive ion modes was employed to ionize the chemical compounds. The detection conditions are listed as follows: scan time 0.2 s, sheath gas flow rate 45, capillary temperature 320 °C, and electrospray current 0.4 μA. Sample data were collected and processed using Xcalibur software.

**Results**

**Screening of CYP450 candidate genes and sequences analysis**

As celastrol is mainly accumulated in the root of T. hypoglaucaum, we screened the CYP450 genes specifically expressed in the roots according to transcriptome data [28]. A total of ten genes with higher expression in the roots were obtained according to the gene expression heatmap shown in Fig. 2. The high correlation between the gene expression specificity and the tissue expression profile of celastrol allowed us to speculate that these genes may be associated with the biosynthesis of celastrol. The coding sequences, the deduced amino acid sequences, calculated molecular masses, and computed isoelectric points of candidate genes are listed in Table S1. Based on the CYP450 candidate sequences screened from the transcriptome data, the 5’ and 3’ specific primers were designed to bind to both ends of the open reading frame (ORF) using Prime Premier 5.0 software, as shown in Supplementary Table S2, and the cDNA first chain obtained by reverse transcription was used as a template for PCR to obtain the full-length of the cDNA sequences.

Multiple sequence alignments showed 37.61% sequence similarity of the ten CYP450 genes from T. hypoglaucaum with two CYP450 genes from Medicago truncatula and Glycyrrhiza inflata, respectively, which were reported to be involved in the biosynthesis of triterpenoid, as shown in Fig. 3A. Functional motif analysis revealed two conserved motifs in the CYP450 genes (Figs. 3B and 3C), where the FxGxRxGxG motif was regarded as the conserved heme binding motif and the ExxR motif was considered important for the sta-
showed in Fig. 5, the retention time and mass spectrum of the formed two new peaks compared with the control group. As CYP712K1 revealed that only one of the candidates, 

CYP712K1, which belong to the CYP71 clan from T. wilfordii [29], and ThCYP712K1, CYP712K4 from M. ilicifolia, and TwCYP712K1, TwCYP712K2 and TwCYP712K3 from T. wilfordii, which belong to the CYP71 clan [29], and have been reported to convert friedelin into polpunonic acid, also called maytenoic acid [28]. By contrast, ThCYP716E53 clustered with CaCYP716E41, which converted oleanolic acid to maslinic acid [31]. ThCYP88H10 clustered with GuCYP88D6, which readily catalyzed the hydroxylation of 30-hydroxy-β-amyrin at C-11 [32].

Functional characterization of CYP450 candidates through heterologous expression in yeast

Seven full-length coding sequence of the candidate CYP450 genes were cloned from the T. hypoglaucum cDNA library. To investigate the function of these genes, we co-expressed each CYP450 with the oxidosqualene cyclase (TwOSC1) and cytochrome P450 reductase (TwCPR3) in lanosterol synthase-deficient yeast [30].

UPLC/Q-TOF-MS analysis of the fermentation products revealed that only one of the candidates, ThCYP712K1 formed two new peaks compared with the control group. As showed in Fig. 5, the retention time and mass spectrum of the two products showed excellent matches to the authentic reference standard of polpunonic acid and 29-hydroxyfriedelin-3-one. The results indicated that ThCYP712K1 mainly catalyzed the oxidation of friedelin at the C-29 position to yield polpunonic acid, a possible biosynthetic precursor of celastrol. For the catalytic efficiency of TwCYP712K1 and ThCYP712K1, we found that ThCYP712K1 accumulated twice as much as TwCYP712K1 in the amount of 29-hydroxyfriedelan-3-one. However, TwCYP712K1 catalyzed to form more polpunonic acid, as shown in Supplementary Figs. S1 and S2.

In vivo experiment of ThCYP712K1

To further verify the function of ThCYP712K1, we examined the activity of ThCYP712K1 through substrate supplementation. ThCYP712K1 was co-expressed with TwCPR3 as a redox partner in lanosterol synthase-deficient yeast. Following the culture of the transgenic yeast strain in medium containing galactose to induce the expression of ThCYP712K1 and TwCPR3, the culture was supplemented with friedelin or 29-hydroxy-friedelan-3-one. Then, ethyl acetate extracts of the cells were analyzed. As shown in Figs. 6A and 6B, when supplemented with 29-hydroxy-friedelan-3-one, ThCYP712K1 converted the substrate into polpunonic acid, as shown in Supplementary Fig. S3. However, no new peak were detected when the cells were supplemented with friedelin compared with the control group, probably because the solubility of friedelin is relatively poor and highly hydrophobic substrates are unable to cross the membrane into the yeast cell.

In vitro enzymatic assays of ThCYP712K1

For in vivo analysis, we extracted microsomes from the
yeast cells and incubated the proteins with friedelin or 29-hydroxy-friedelan-3-one. As shown in Figs. 6A and 6B, the target peak was detected in the reaction mixture with ThCYP712K1, implying that the enzyme converted 29-hydroxy-friedelan-3-one to polpunonic acid \textit{in vitro}, as shown in Supplementary Fig. S4. The consistent result of the \textit{in vivo} and \textit{in vitro} enzyme assays finally demonstrated that ThCYP712K1 catalyzed the oxidation of friedelin to gener-

Fig. 3 Alignment of amino acid sequences and conserved motifs in candidate CYP450s from \textit{T. hypoglauceum} and other plants. (A) Multiple sequence alignment and conserved motifs of ThCYP712K1 and other CYP450s; (B) ExxR motif; and (C) FxxGxR-CxG motif (x refers to variable amino acids)
ate 29-hydroxy-friedelan-3-one and polpunonic acid in celastrol biosynthesis.

Exploring the substrate specificity of ThCYP712K1

In vivo yeast feeding assay of ThCYP712K1 recombinant yeasts were performed using several triterpenoids, such as lupeol, oleanolic acid, ursolic acid, betulinic acid, and α-amyrin. However, no corresponding products were detected by UPLC/Q-TOF-MS, which indicated that ThCYP712K1 has relatively strict substrate specificity, as shown in supplementary Fig. S5.

Discussion

T. hypoglaucum is known to contain a diversity of triterpenes, and celastrol is one of the most valuable compounds isolated from this plant [34]. In this study, we focused on the CYP450 genes that catalyze the formation of C-29 carboxyl group of celastrol. As celastrol is mainly concentrated in the roots, especially in the root bark, we screened CYP450 genes from T. hypoglaucum transcriptome data based on their transcriptional levels in different tissues. As a result, ten candidate genes with tissue-specific expression in the roots were screened out, in which seven were cloned and functionally expressed in yeast. Results showed that only one of the candidates catalyzed the oxidation of friedelin to form polpunonic acid. Triterpenes account for a large proportion of plant secondary metabolites in nature, and the complexity and diversity of their structures is closely related to the diverse functions of CYP450s. To date, many CYP450 genes have been isolated and characterized, including the members of the CYP71D, CYP72A and CYP716A subfamilies, which have been reported to be involved in the formation of the carbon skeleton of triterpene biosynthesis [32, 35-38]. In order to further speculate on the catalytic function of these ThCYP450s, the candidates were compared with CYP450s related to triterpene biosynthesis from other plants. Phylogenetic tree analysis showed that ThCYP712K1 clustered close to the CYP712 family, where the amino acid sequence similarity of ThCYP712K1 and TwCYP712K1 was as high as 98%. The functional characterization in yeast revealed that ThCYP712K1 converted friedelin to finally form polpunonic acid via the intermediate 29-hydroxyfriedelan-3-one. However, the other CYP450 genes showed no activity towards friedelin, but they might have oxidative effects on other compounds, which need further investigation in the subsequent work. In vitro enzyme activity assays revealed that ThCYP712K1 utilized 29-hydroxyfriedelan-3-one as a sub-

Fig. 4  Phylogenetic analysis of CYP candidates and CYP450 genes from other species. A phylogenetic tree was constructed using MEGA 7.0 software using the neighbor-joining method with 1000 bootstrap iterations
strate to produce polpunonic acid, indicating the oxidation of friedelin at the C-29 position catalyzed by the CYP enzyme. However, when we use friedelin as a substrate, we did not detect any production peak, which may result from the poor solubility of friedelin. For the function of ThCYP712K1, we have conducted a relatively complete study. In future work, the mechanisms of the CYP450 that catalyzes the hydroxylation and carboxylation at the C-29 position of friedelin will

Fig. 5 UPLC/Q-TOF-MS analysis of the products in yeast strains harbouring ThCYP712K1 chromatograms of the products in yeast strains harbouring ThCYP712K1. (A) Peak 1: 29-hydroxyfriedelan-3-one; (B) Peak 2: polpunonic acid; (C) EI-MS spectra of the 29-hydroxyfriedelan-3-one produced by the yeast strain the authentic standard; (D) EI-MS spectra of the polpunonic acid produced by the yeast strain and the authentic standard; and (E) The proposed biosynthetic steps catalyzed by ThCYP712K1
be further investigated. For example, molecular docking and site-directed mutagenesis experiments will be used to identify the key amino acid residues regulating the formation of 29-hydroxy-friedelan-3-one and polpunonic acid, so that 29-hydroxy-friedelan-3-one will be transformed into polpunonic acid as much as possible, with the aim of ultimately improving the catalytic efficiency of enzyme.

In summary, our study confirmed the function of ThCYP712K1 as the enzyme that catalyzes the C-29 oxidation of friedelin to form polpunonic acid in the biosynthetic pathway of celastrol in T. hypoglaucum. Although CYP450s with related functions have been reported in other species, there have been no reports on similar CYP450s in T. hypoglaucum. To the best of our current knowledge, this is the first study to identify the corresponding CYP450 from T. hypoglaucum and its function has been verified both in vivo and in vitro. In addition, as T. hypoglaucum is less toxic than T. wilfordii [39], this study is helpful for further exploring the biosynthesis pathway of celastrol in T. hypoglaucum. In future studies, an engineered yeast cell factory will be established for high-yield production of polpunonic acid.

**Supplementary Information**

All the supporting information of this paper can be requested by sending E-mail to the corresponding author.

**References**


Cite this article as: CHEN Xiao-Chao, LU Yun, LIU Yuan, ZHOU Jia-Wei, ZHANG Yi-Feng, GAO Hai-Yun, LI Dan, GAO Wei. Identification of a cytochrome P450 from Tripterygium hypoglaucum (Levl.) Hutch that catalyzes polpunonic acid formation in celestrol biosynthesis [J]. Chin J Nat Med, 2022, 20(9): 691-700.