

Probing the Catalytic Promiscuity and Enhancing the Efficiency of CYP725A4 with a Taxadiene Substrate Mimic

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Cite This: <https://doi.org/10.1021/acscatal.6c04534>



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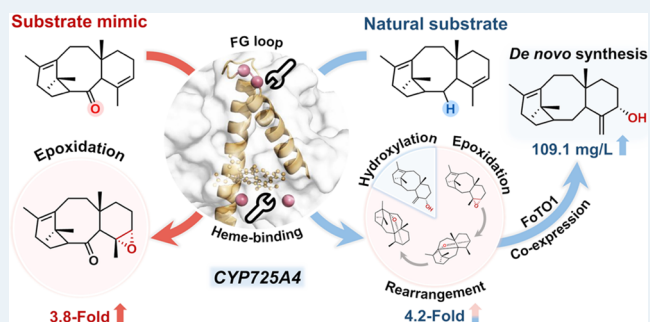
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ABSTRACT: The catalytic promiscuity of cytochrome P450 CYP725A4 severely impedes paclitaxel biosynthesis, as it generates taxadien-5 α -ol (T-5 α -ol) alongside numerous by-products. Despite diverse proposed mechanisms and the reported role of facilitator of taxane oxidation (FoTO1) in promoting T-5 α -ol formation, conclusive evidence elucidating the catalytic promiscuity of CYP725A4 remains elusive. Here, we report a substrate mimic strategy using taxadien-2-one (taxadienone), which is selectively epoxidized by CYP725A4 at the C4=C5 double bond, suggesting epoxidation as its major catalytic pathway. This notion was then confirmed by the successful identification of the long-observed yet structurally uncharacterized major taxadiene oxidation product as 4(5) α -epoxy-taxadiene, which can spontaneously rearrange in solution. Furthermore, the minor T-5 α -ol produced by CYP725A4 arises from a concurrent hydroxylation pathway rather than from epoxidation, and efficient isomerization of 4(5) α -epoxy-taxadiene to T-5 α -ol by FoTO1 is required to achieve improved product selectivity. Employing the promiscuity-free taxadienone as a probe substrate eliminated analytical complications and led to variant M3, which shows markedly enhanced catalytic activity through optimized heme-binding and enhanced FG loop flexibility. Finally, co-expressing the plant-derived FoTO1 with the CYP725A4 variant M3 in engineered *Escherichia coli* increased T-5 α -ol production to 109.1 mg/L in a 5 L bioreactor. Collectively, this strategy provides crucial insights into CYP725A4 catalytic promiscuity and enables more efficient directed evolution, thereby facilitating downstream paclitaxel pathway optimization.

KEYWORDS: paclitaxel, P450, epoxidation, hydroxylation, directed evolution



INTRODUCTION

Cancer remains a major global health threat with a substantial disease burden.¹ Paclitaxel is a highly effective anticancer agent widely applied in the treatment of breast, ovarian, pancreatic, and prostate cancers.^{2–5} Although industrial production employs semi-synthesis to supply clinical demand, it still heavily depends on plant-derived starting materials.^{6–8} Advances in synthetic biology have unlocked significant potential for the heterologous biosynthesis of natural products.^{9–11} Paclitaxel and its key intermediate baccatin III have been successfully synthesized in heterologous systems such as *Nicotiana benthamiana*^{12–14} and *Saccharomyces cerevisiae*.¹⁵ However, a series of P450-mediated oxygenation events on the taxadiene (**1**) scaffold exhibit high promiscuity and low catalytic activity, leading to severely limited yields in heterologous paclitaxel biosynthesis.^{16–19}

CYP725A4 is the first cytochrome P450 enzyme involved in the paclitaxel biosynthetic pathway (Scheme 1), catalyzing the oxygenation of taxadiene (**1**) to yield taxadien-5 α -ol (T-5 α -ol, **5**).^{20,21} When heterologously expressed in taxadiene-producing chassis cells, CYP725A4 generates a diverse array of oxygenated taxanes, with the desired product T-5 α -ol (**5**)

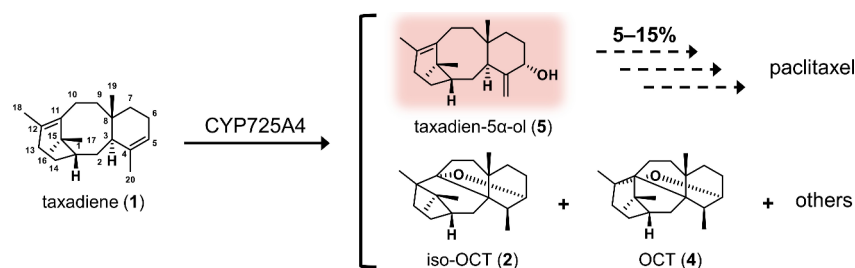
accounting for merely 5–15% within the total oxygenated products.^{22–24} Other products, such as 5(12)-oxa-3(11)-cyclotaxane (OCT, **4**) and 5(11)-oxa-3(11)-cyclotaxane (iso-OCT, **2**), are believed not to serve as precursors in paclitaxel biosynthesis. Consequently, the insufficient yield of T-5 α -ol (**5**) presents a major bottleneck in the subsequent biosynthetic steps.²⁵ A recent study has demonstrated that co-expression of the facilitator of taxane oxidation (FoTO1) in *N. benthamiana* effectively suppresses the formation of dead-end products OCT (**4**) and iso-OCT (**2**), resulting in T-5 α -ol (**5**) and its over-oxidized product (4 α ,20-epoxy-taxadien-5 α -ol) becoming the major products.¹² However, the inherent catalytic promiscuity of

Received: June 12, 2026

Revised: July 29, 2026

Accepted: August 14, 2026

Scheme 1. Catalytic Reaction of CYP725A4 during the Biosynthesis of Paclitaxel



CYP725A4 is still unclear, and its low catalytic activity remains a major challenge.

Substantial research efforts have been directed toward unraveling the molecular mechanisms underlying the catalytic promiscuity of CYP725A4. In the originally proposed radical rebound-mediated mechanism (Figure 1A), the poor regioselectivity of CYP725A4 toward taxadiene (1) leads to the formation of diverse oxygenated products, with T-5α-ol (5) formed via oxygen rebound at the C5 site.^{20,21,26} An alternative mechanism suggests the formation of an epoxide intermediate during the CYP725A4-catalyzed oxygenation of taxadiene (1) (Figure 1B), from which OCT (4), iso-OCT (2), and T-5α-ol (5) arise through its spontaneous rearrangement.^{24,27–29} A structure-based computational study has further investigated competing epoxidation and O-protonation pathways in the oxygenation of taxadiene (1) (Figure 1C). A key distinction lies in the generation of side products OCT (4) and iso-OCT (2) through the protonated substrate cation intermediate facilitated by CYP725A4.³⁰ However, neither of the latter two proposed mechanisms has confirmed the existence of the epoxide intermediate (Figure 1B,C), conclusive experimental evidence to fully elucidate the catalytic promiscuity of CYP725A4 is still lacking.

Despite structural characterization and extensive mutagenesis studies on CYP725A4, current mechanistic insights are still insufficient to guide the identification of variants with higher catalytic activity or improved product selectivity.^{26,28,30} While ancestral sequence reconstruction provides valuable insights into functionally critical residues,³¹ the screening of mutant libraries remains challenging due to the lack of high-throughput screening methods, combined with the structural diversity and environmental lability of oxygenated products.²⁴ Therefore, establishing an effective screening strategy to circumvent catalytic promiscuity is critical for enabling efficient directed evolution and obtaining CYP725A4 variants with enhanced catalytic efficiency.

In this study, we uncovered taxadien-2-one (taxadienone, 6) from a series of taxadiene (1) structural analogs as a selectively epoxidized probe substrate for investigating CYP725A4-mediated oxygenation. Using this substrate mimic, we elucidated the catalytic mechanism underlying CYP725A4's promiscuity. We provided experimental evidence that the mechanism proceeds via a major, unstable epoxidation product of taxadiene (1), which undergoes subsequent rearrangements to generate diverse by-products, while the minor T-5α-ol (5) is produced via a concurrent pathway (Figure 1D). Furthermore, FoTO1 can isomerize the epoxidation product into T-5α-ol (5) to improve selectivity. Capitalizing on this insight, we implemented taxadienone (6) as a well-defined model substrate that circumvents analytical complications arising from the catalytic

promiscuity of CYP725A4. This approach enabled precise quantifications in the oxidative activity of CYP725A4 and thus established a reliable platform for sequence- and structure-guided enhancement of its catalytic efficiency. Finally, heterologous co-expression of the optimal CYP725A4 variant with FoTO1 in *Escherichia coli* significantly enhanced T-5α-ol (5) production in vivo.

MATERIALS AND METHODS

Plasmid Construction

E. coli TOP10 and BL21(DE3) strains (Novagen, Darmstadt, Germany) were utilized for cloning and expressing respectively. The plasmids pACYCDuet-1 and pRSFDuet-1 (Novagen, Darmstadt, Germany) were employed as vectors for the integration and expression of the target proteins. All proteins employed in this study are comprehensively detailed in Table S4. The corresponding genes were cloned into vectors using the ClonExpress Ultra One Step Cloning Kit (Vazyme, Nanjing, China). Specifically, the genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), isopentenyl diphosphate isomerase (IDI), geranylgeranyl diphosphate synthase (GGPPS), taxadiene synthase (TS), CYP725A4 and cytochrome P450 reductase (CPR) were assembled on the pACYCDuet-1 vector.³² CYP725A4 and CPR were fused into a single chimera (CYP725A4-CPR) using a GSTGS linker peptide. The N-terminal 74 amino acids of CPR and 24 amino acids of CYP725A4 were truncated, and a small peptide "MALLLAVF" from bovine was added to the N-terminal of truncated CYP725A4 to improve the protein solubility during intracellular expression.²² In a separate construct, the gene of FoTO1 was cloned into the pRSFDuet-1 vector.

Media and Culture Conditions

To obtain engineered strains for de novo T-5α-ol (5) synthesis, the recombinant plasmids (pACYCDuet1-DXS-IDI-TS-GGPPS-(CYP725A4-CPR), pRSFDuet-1-FoTO1) were co-transformed into *E. coli* BL21(DE3) competent cells via heat-shock. Transformants were selected on lysogeny broth (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 2% (w/v) agar) containing the corresponding antibiotics. A single well-grown colony was selected, inoculated into 4 mL LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with kanamycin (50 mg/L) and chloramphenicol (34 mg/L), and grown for 12 h at 37 °C with shaking at 200 rpm to prepare the seed culture. For T-5α-ol (5) production, the seed culture was inoculated at 1% (v/v) into 50 mL of Terrific Broth (TB) medium (12 g/L tryptone, 24 g/L yeast extract, 2.31 g/L KH₂PO₄, 12.54 g/L K₂HPO₄, and 20 g/L glycerol) with antibiotics in a 250 mL flask. The culture was incubated at 37 °C with shaking at 200 rpm until the OD₆₀₀ reached 1.0 (approximately 3.5–4 h). The temperature was lowered to 18 °C for 20 min, after which protein expression and heme supply were induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.2 mM δ-aminolevulinic acid (ALA). Induction was then continued for 72 h at 18 °C.

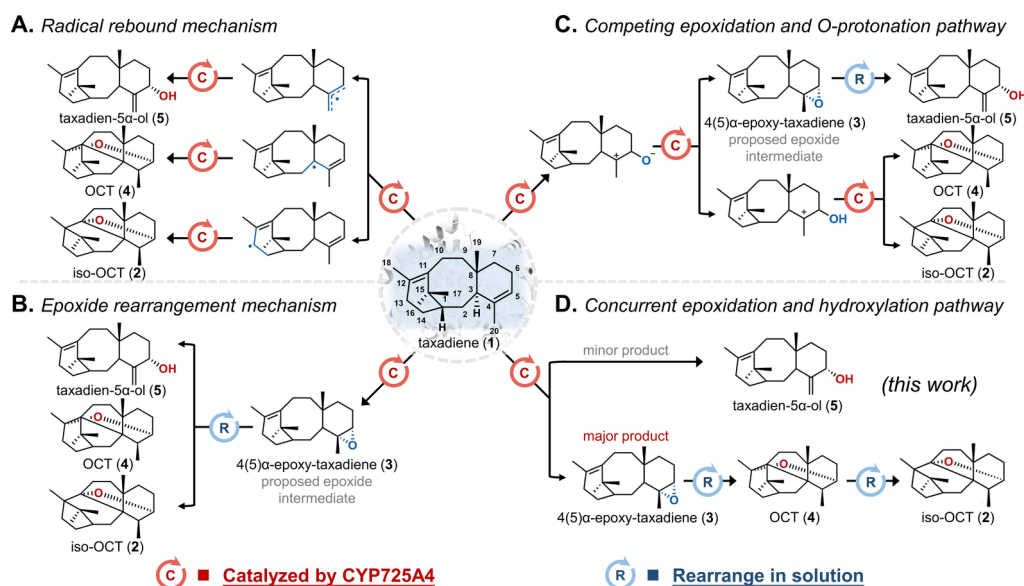


Figure 1. Proposed reaction mechanisms for CYP725A4-catalyzed oxygenation of taxadiene (1). (A) Radical rebound mechanism.^{20,21,26} (B) Epoxide rearrangement mechanism.^{24,29} (C) Competing epoxidation and O-protonation via a zwitterionic intermediate.³⁰ (D) Concurrent epoxidation and hydroxylation pathway (proposed in this work).

For fed-batch cultures, the seed culture was transferred to a 2 L shake flask containing 300 mL LB medium, and grown at 37 °C with shaking at 200 rpm until the OD₆₀₀ reached 2.0. The secondary seed culture was then inoculated in a 5 L bioreactor with 3 L fermentation medium (20 g/L glycerol, 10 g/L tryptone, 5 g/L yeast extracts, 5 g/L Na₂HPO₄·12H₂O, 0.7 g/L Na₂SO₄, 3.4 g/L KH₂PO₄, 0.25 g/L MgSO₄, 2.7 g/L NH₄Cl, 28 mg/L FeSO₄·7H₂O, and 0.2% (w/v) anti-foam). The pH of fermentation was automatically maintained at 6.9 by adding sulfuric acid (1 M) or ammonia (50%). The fermentation was conducted at 37 °C with an initial stirring speed of 200 rpm and an air flow rate of 1 vvm (3 L/min). Dissolved oxygen (DO) was maintained above 25% by automatically increasing the agitation speed in increments of 3 rpm/10 s. When OD₆₀₀ reached 15–20, the temperature was lowered to 18 °C to induce protein expression, consistent with the shake-flask cultivation procedure. Once the DO rose above 60%, a feed solution (250 g/L glycerol, 120 g/L tryptone, and 60 g/L yeast extract) was supplied at a rate of 1.5 L/h to bring the DO back to approximately 30%. Throughout the fermentation, samples were taken every 8 h for OD₆₀₀ measurement and product analysis.

Synthesis of Bio-oxidation Products

Compounds 2–5 and 7–9 were isolated from *in vitro* biotransformation reactions using *E. coli* cell lysate expressing CYP725A4 as the sole catalyst, compound 10 was isolated from *in vitro* biotransformation reactions using *E. coli* cell lysate expressing FoTO1. To control pH fluctuations during the reaction, BstFDH (0.2 mM NADP⁺, 20 g/L lyophilized BstFDH, 1%, w/v sodium formate) and BmGDH (0.2 mM NADP⁺, 2 g/L lyophilized BmGDH, 1%, w/v glucose) were used for cofactor recycling. Detailed methods and characterization data of all compounds are shown in supplementary information. The unstable compound 3 readily undergoes spontaneous rearrangement to compounds 2 and 4. To minimize this rearrangement, all preparation and purification procedures were conducted under basic conditions, and the purified compound was lyophilized for storage.

Site-Directed Mutagenesis of CYP725A4 and Mutant Screening

Site-directed mutagenesis was performed by a whole-plasmid PCR method using PrimeSTAR[®] HS (Premix) (TaKaRa). All mutagenesis

primers were commercially synthesized by Tsingke Biotechnology Co., Ltd. (Shanghai, China). The polymerase chain reaction (PCR) was performed using the CYP725A4-CPR gene as the template. Each 20 μ L reaction contained 10 μ L of Premix PrimeSTAR HS, 2 μ L of primer mix (forward and reverse), 1 μ L of template plasmid, and 7 μ L of deionized water. The PCR procedure was performed as follows: initial denaturation at 95 °C for 5 min, then 35 cycles of 98 °C for 10 s, 55 °C for 5 s, 72 °C for 8 min, and a final extension at 72 °C for 5 min. The PCR products were further digested with 1 μ L Dpn I (New England Biolabs) at 37 °C for 2 h to remove the template DNA. The resulting plasmids—pACYCDuet-1 containing either wild-type or mutant CYP725A4-CPR were then transformed into *E. coli* BL21(DE3) competent cells. All the mutations were verified by DNA sequencing. For functional screening, transformed colonies were cultured and induced for protein expression at 22 °C. Cells were collected by centrifugation and resuspended in KPB (100 mM, pH 7.4). A 400 μ L reaction system containing cell lysate (25 g/L wet cell), taxadienone (1 mM), NADPH (10 mM), methanol (5%, v/v) in KPB was incubated at 25 °C with shaking at 1000 rpm for 30 min. Products were extracted using ethyl acetate, and the organic layer was collected after centrifugation, dried, and subjected to GC-MS analysis.

Expression and Purification of FoTO1 Protein

The recombinant plasmid pRSFDuet-FoTO1 was transformed into competent cells. One fresh colony was then inoculated into 4 mL of LB medium supplemented with kanamycin (50 μ g/mL) and incubated at 37 °C and 200 rpm for 12 h. The seed culture was inoculated at 1% (v/v) into a shake flask containing 100 mL TB medium supplemented with kanamycin (50 μ g/mL) and incubated at 37 °C and 200 rpm. When the OD₆₀₀ reached 0.6–0.8 (approximately 2.5–3 h), the culture temperature was adjusted to 16 °C. After cooling for 20 min, IPTG was added to a final concentration of 0.15 mM to induce protein expression. Incubation was continued for 24 h, and the cells were harvested by centrifugation at 10,000 rpm for 5 min.

The harvested cells were resuspended in lysis buffer (25 mM Hepes, pH 7.5, 500 mM NaCl, 20 mM imidazole) and lysed by sonication on ice. The lysate was centrifuged at 12,000 rpm for 40 min at 4 °C, and the supernatant was filtered through a 0.22 μ m membrane filter. The filtrate was applied to a Ni-NTA affinity column pre-equilibrated with

lysis buffer. The column was washed with wash buffer (25 mM Hepes, pH 7.5, 500 mM NaCl, 120 mM imidazole) to remove nonspecifically bound proteins. FoTO1 was then eluted with elution buffer (25 mM Hepes, pH 7.5, 500 mM NaCl, 350 mM imidazole). The sample was exchanged three times by ultrafiltration using storage buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM DTT, 5% glycerol) to remove the majority of imidazole. The concentration of purified FoTO1 was determined by the BCA method, and the protein was stored at -80°C .

GC-MS Detection Method

For the analysis of fermentation broths and reaction mixtures, samples were extracted with an equal volume of ethyl acetate containing 5 mg/L octadecane as internal standard, followed by vortexing for 30 min. The organic phase was separated by centrifugation (13,000 rpm, 5 min, 4°C) and dried with anhydrous sodium sulfate. GC-MS analysis was performed using an Rtx-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) on GCMS-QP2030 system (Shimadzu, Kyoto, Japan) with helium as the carrier gas. Electron ionization was employed for mass spectral detection. A 1 μL sample was injected in split mode (split ratio 15:1). The injector and column oven were set at 250°C and 80°C , respectively. The injector temperature was set to 250°C , and the initial column temperature was held at 80°C for 1 min, then increased to 220°C at $15^{\circ}\text{C}/\text{min}$ and held for 5 min, followed by a ramp to 280°C at $20^{\circ}\text{C}/\text{min}$ and a final hold for 1 min. For oxygenated products that could not be obtained in sufficient quantity, they were quantified by utilizing the calibration curve of taxadienone (6), considering their structural and mass similarity.

Construction of the Enzyme–Substrate Complex System

The initial coordinates of CYP725A4 were derived from its crystal structure (PDB ID: 8X1W).³⁰ Mutants were generated in ChimeraX³³ by manual residue replacement, selecting the highest-probability amino acid conformer for each substitution. The structure of taxadiene (1) (PubChem CID: 443484) was acquired from PubChem. Complex structures for the mutants with substrate were generated through AutoDock Vina docking³⁴ and served as initial structures for restrained MD simulations. Protonation states of ionizable residues were assigned using PROPKA³⁵ at pH 7.4. The Amber ff14SB force field³⁶ was applied for the protein. Force field parameters for compound 1 (Cpd 1) covalently linked to cysteine were adopted from previous work,³⁷ with Cpd 1 coordinates aligned to the protein-bound heme. Each system was solvated in a cubic TIP3P water box³⁸ and neutralized with explicit Cl^{-} ions. Atomic charges for the substrate were derived using the RESP method³⁹ based on electrostatic potentials calculated at the DFT level with ORCA,⁴⁰ and processed through Multiwfn.⁴¹ Topology files for the substrates were generated using the Sobtop toolkit (Tian Lu, Sobtop, Version [1.0 (dev 3.1)], <http://sobereva.com/soft/Sobtop>, accessed on November 1, 2024) based on the General Amber Force Field (GAFF).⁴²

Molecular Dynamics (MD) Simulation

All simulations were performed using GROMACS 2023.⁴³ Energy minimization was conducted via the steepest descent method and the conjugate gradient method to eliminate atomic clashes. Systems were then equilibrated for 5 ns under positional restraints applied to the protein and substrate, with hydrogen bonds constrained via SHAKE.⁴⁴ Long-range electrostatic interactions were treated with Particle Mesh Ewald (PME)⁴⁵ and used 10 Å cutoffs for both Coulombic and van der Waals interactions. The system temperature was maintained at 298.15 K using the V-rescale thermostat,⁴⁶ and pressure was controlled at 1.0 atm using the Berendsen barostat⁴⁷ in equilibrium stage and Parrinello–Rahman barostat⁴⁸ in MD simulations. A 1 fs timestep was employed during equilibration with periodic boundary conditions. All MD simulations were performed without positional restraints (retaining only hydrogen bond constraints) using a 2 fs timestep. For each system, two independent 200 ns replicates were conducted under periodic boundary conditions. All analyses were conducted using the

full-length trajectories from both independent simulations. Representative structures were extracted from stable trajectory segments based on minimal average backbone RMSD.

RESULTS

Substrate-Mimetic Probe Reveals the Major Catalytic Pathway of CYP725A4

In the CYP725A4-catalyzed oxygenation of taxadiene (1) (m/z 272), four monooxygenated products with the mass-to-charge ratio (m/z) of 288 were detected, reflecting pronounced product promiscuity (Figure 2A). Among these, three compounds exhibited mass spectra consistent with structurally characterized iso-OCT (2), OCT (4), and T-5 α -ol (5).^{17,24,26,29} Notably, the major monooxygenated product—denoted UOTX (3), an unstable oxygenated taxane with undetermined structure, was also detected (Figure S1), which was previously observed only in overlay fermentation.^{31,49} Its pronounced instability leads to rapid degradation during product isolation and purification, thereby preventing structural characterization.⁵⁰

While extensive studies have established the importance of enzyme structure in catalysis, the substrate architecture constitutes an equally critical component that fundamentally governs the catalytic efficiency and selectivity.⁵¹ Substrate engineering has thus emerged as a powerful complementary strategy to modulate the activity and selectivity of various enzymes.^{52–55} Owing to the distinct positions of the oxidized double bond, iso-taxadiene was insufficient to account for the product promiscuity observed for taxadiene.^{24,30} To probe how substrate structure influences the catalytic promiscuity of CYP725A4, we designed a series of taxadiene analogs with site-specific modifications and varied oxidation states, guided by the structural features of paclitaxel (Figure S2). We further filtered out molecules that were unlikely to bind effectively based on molecular docking analysis, and selected the remaining analogs for experimental testing. Surprisingly, in contrast to taxadiene (1), the oxygenation of taxadienone (6) (m/z 286, a chemical precursor to taxadiene (1) with a C2-ketone group)^{56–58} proceeded with high product selectivity, yielding a single monooxygenated product 7 (m/z 302) during the reaction (Figure 2B). Compound 7 exhibited high stability, facilitating its isolation for further characterization. Through 2D-NMR analysis, compound 7 was identified as 4(5) α -epoxy-taxadienone in which the site- and stereo-selectivity are consistent with the proposed epoxide intermediate derived from taxadiene (1).²⁹ This result demonstrates the capability of CYP725A4 to catalyze epoxidation at the C4=C5 double bond of taxane scaffold, suggesting that the major oxygenated product UOTX (3) from taxadiene (1) may likewise possess an epoxide structure.

Moreover, *in vitro* assays revealed that the degradation of UOTX (3) can be mitigated by optimizing the reaction conditions (Figure S3), making it possible to characterize the structure of UOTX (3). To minimize acid-accelerated decomposition of UOTX (3), the CYP725A4-catalyzed oxygenation of taxadiene (1) was adjusted to pH 7.4–8.0. Furthermore, basic alumina column was employed for product purification instead of conventional silica gel column. Subsequent 2D-NMR analysis confirmed the purified enzyme-catalyzed product UOTX (3) as the previously hypothesized epoxide intermediate 4(5) α -epoxy-taxadiene (3) (Figure 2A), filling a critical gap in the characterization of taxadiene (1) oxygenation products catalyzed by CYP725A4. This finding confirms that the major

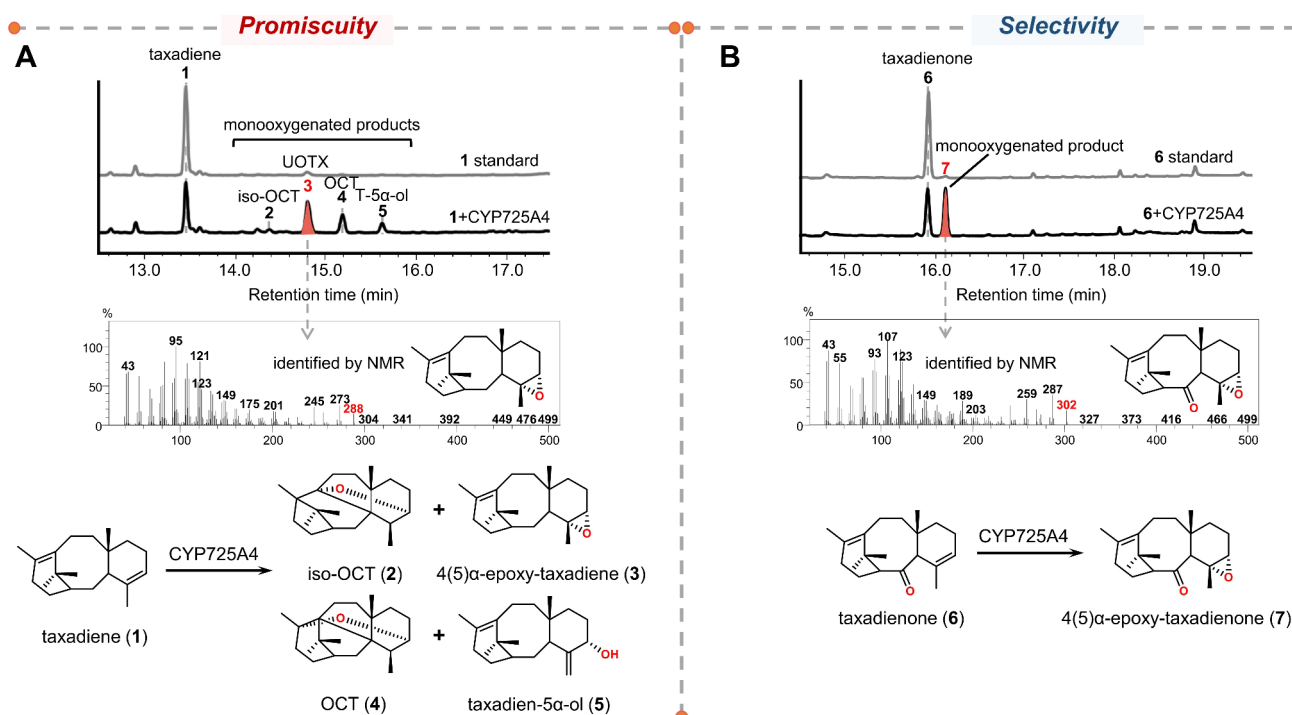


Figure 2. Product analysis of CYP725A4-catalyzed reactions toward taxadiene (1) and taxadienone (6). (A) Total ion chromatogram (TIC) of the oxygenated products of taxadiene (1) obtained by GC-MS analysis, with the mass spectrum corresponding to the previously uncharacterized product 3. (B) TIC of the oxygenated products of taxadienone (6) obtained by GC-MS analysis, with the mass spectrum corresponding to product 7. The epoxidation products 3 and 7 were identified as 4(5) α -epoxy-taxadiene and 4(5) α -epoxy-taxadienone, respectively (highlighted in red), through NMR structural elucidation and confirmed by high-resolution EI-MS (Figures S20, S26–S37, Tables S3, S5, and S6). The structures of additional oxygenated products, including iso-OCT (2), OCT (4), and T-5 α -ol (5), were assigned based on mass spectral and ^1H NMR data¹⁶ (Figures S20, S22–S24, and Table S1).

catalytic pathway of CYP725A4 is to catalyze epoxidation of the C4=C5 double bond on the taxadiene scaffold, and substrate modification can enable the highly selective epoxidation of CYP725A4.

Elucidating the Catalytic Promiscuity during the Oxygenation of Taxadiene

Based on the characterization of oxygenated products of taxadiene (1) and taxadienone (6) catalyzed by CYP725A4, we propose that the observed catalytic promiscuity results from two main factors: competing oxidative activities (epoxidation vs hydroxylation) toward taxadiene (1), and the inherent instability of its epoxide intermediate, which promotes the formation of diverse by-products (Figure 3A). To investigate the spontaneous interconversion among the promiscuous products, purified 4(5) α -epoxy-taxadiene (3) was incubated in aqueous solution at pH 7.0. GC-MS TICs revealed a time-dependent decrease in the concentration of 4(5) α -epoxy-taxadiene (3), accompanied by an increase in OCT (4) and a gradual rise in iso-OCT (2) (Figure 3B). Previous studies have demonstrated that OCT (4) undergoes spontaneous rearrangement into iso-OCT (2).²⁴ These findings support a sequential spontaneous rearrangement pathway in which 4(5) α -epoxy-taxadiene (3) converts to OCT (4), which subsequently rearranges into iso-OCT (2). This rearrangement is accelerated under acidic conditions, with complete conversion of 1 mM 4(5) α -epoxy-taxadiene (3) to iso-OCT (2) at pH 1.0 (Figure S4). Notably, no accumulation of T-5 α -ol (5) was detected throughout this

rearrangement process. Furthermore, during the chemical epoxidation of taxadiene (1), the aforementioned product rearrangement was also observed to be highly susceptible to catalytic conditions (Figure S5). Compared with the enzymatic reaction, no significant formation of hydroxylation products was detected. In contrast, the mono-oxygenated product derived from the substrate mimic taxadienone (6) exhibits high stability and does not undergo rearrangement. To investigate whether CYP725A4 mediates the isomerization of the epoxidation product to the hydroxylation product, we performed enzymatic oxygenation of 4(5) α -epoxy-taxadiene (3) using CYP725A4. No conversion to T-5 α -ol (5) was observed; only trace quantities of dioxygenated product 8 were detected, which was subsequently identified as 4 β ,19-ether-taxadien-5 α -ol by 2D-NMR (Figure 3C). This compound was also detected in the taxadiene (1) oxygenation (Figure S6). These findings rule out the possibility that the hydroxylation product in the CYP725A4 reaction system is generated via an epoxide intermediate. Moreover, previous kinetic studies on taxadiene (1) and its isomer have proposed a direct hydroxylation mechanism *via* radical rebound.^{20,21} Therefore, we propose that T-5 α -ol (5) is produced via a hydroxylation pathway concurrent with the epoxidation pathway during CYP725A4 catalysis.

To investigate potentially enzymatic interconversion among other oxygenated products of taxadiene (1), *in vitro* assays were conducted using T-5 α -ol (5), iso-OCT (2), and OCT (4) as substrates for CYP725A4. GC-MS analysis demonstrated that each substrate yielded a selective dioxygenated product

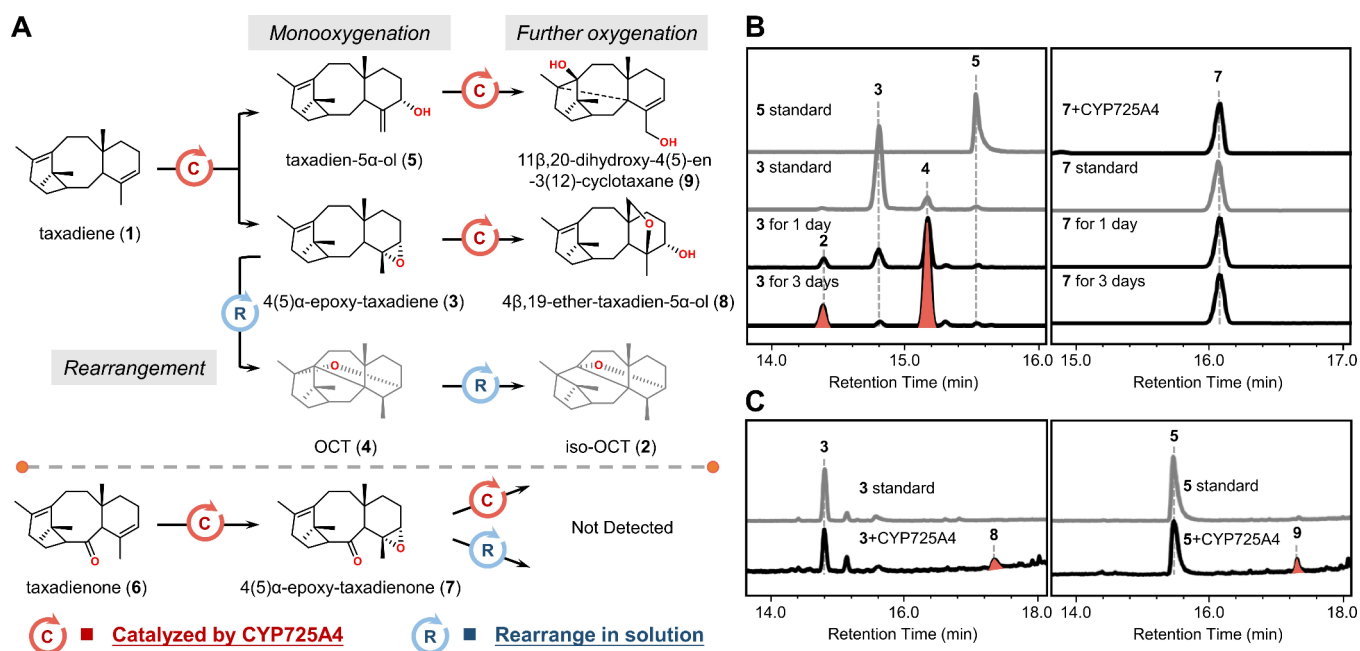


Figure 3. Elucidating the catalytic promiscuity of CYP725A4. (A) Reaction pathways for CYP725A4-catalyzed oxygenation of taxadiene (1) and taxadienone (6). (B) Spontaneous rearrangement of 4(5)α-epoxy-taxadiene (3) in aqueous solution (pH 7.0) as detected by GC-MS TICs. Over time, 4(5)α-epoxy-taxadiene (3) spontaneously rearranges into OCT (4) and a minor amount of iso-OCT (2), while no increase in T-5α-ol (5) is detected. No further oxygenation or rearrangement products were detected for 4(5)α-epoxy-taxadienone (7). (C) GC-MS TICs showing the CYP725A4-catalyzed conversion of the monooxygenated products at m/z 288, 4(5)α-epoxy-taxadiene (3) and T-5α-ol (5), into the corresponding dioxygenated products at m/z 304, 4β,19-ether-taxadien-5α-ol (8), and 11β,20-dihydroxy-4(5)-en-3(12)-cyclotaxane (9). The structures of the dioxygenated products 8 and 9 were characterized by NMR in this study (Figures S20, S38–S51, Tables S7, and S8).

(m/z 304), with no detectable interconversion among these compounds by CYP725A4 (Figure S7). The dioxygenated product 9 generated from T-5α-ol (5) was identified as 11β,20-dihydroxy-4(5)-en-3(12)-cyclotaxane by 2D-NMR (Figure 3C), which is structurally distinct from the previously reported over-oxidized product of T-5α-ol (5)—4α,20-epoxy-taxadien-5α-ol.¹² The further oxygenation products of iso-OCT (2) and OCT (4) were not isolated for structural characterization due to insufficient yields, although multiple structures of their dioxygenated products have been reported previously.^{16,59} These findings indicate that the diverse oxygenated products of taxadiene (1) generated by CYP725A4 can be categorized into three distinct mechanistic pathways: monooxygenation, rearrangement, and further oxygenation (Figure 3A).

Product Selectivity of CYP725A4 Controlled by FoTO1 Isomerization

To improve product selectivity in taxadiene (1) oxygenation, we introduced a reported nuclear transport factor 2 (NTF2)-like protein, FoTO1.¹² Building on our elucidated pathway for the promiscuous oxygenated products generated by CYP725A4, we further investigated the specific role of FoTO1 in enhancing selectivity. Initially, the addition of purified FoTO1 to an in vitro reaction system of CYP725A4-catalyzed oxygenation of taxadiene (1) successfully suppressed the formation of iso-OCT (2), 4(5)α-epoxy-taxadiene (3), and OCT (4), while increasing the yield of T-5α-ol (5) (Figure 4A). This result indicated the absence of the spontaneous rearrangement of the major oxygenated product 3, leading us to propose that FoTO1 likely acts by converting the epoxidation product 3.

Indeed, using purified 4(5)α-epoxy-taxadiene (3) as a substrate, we verified that FoTO1 isomerizes 3 to T-5α-ol (5). We further ruled out the possibility that FoTO1 converts the starting substrate taxadiene (1), or the rearrangement products iso-OCT (2) and OCT (4) (Figure S8). Moreover, a direct protein-protein interaction between FoTO1 and CYP725A4 has previously been reported,¹² which shortens the distance for transferring 4(5)α-epoxy-taxadiene (3) from the catalytic site of CYP725A4 to FoTO1 for subsequent isomerization to T-5α-ol (5). Collectively, these results support a mechanism in which CYP725A4 catalyzes the formation of both the epoxidation product 3 and the hydroxylation product 5, while FoTO1 efficiently captures and converts the main product 3 into the desired product 5. Such coupling effectively suppresses the spontaneous rearrangement of 4(5)α-epoxy-taxadiene (3), thereby contributing to product selectivity during paclitaxel biosynthesis (Figure 4B). The concurrent reporting in two pre-prints of FoTO1 as an epoxide isomerase further corroborates our conclusions.^{60,61}

To further validate the novel function of FoTO1 as an epoxide isomerase, we performed the same reaction assay using the substrate mimic taxadienone (6) (Figure 4C). Under the sequential catalysis of CYP725A4 and FoTO1, taxadienone (6) was converted into a single new product 10. When 4(5)α-epoxy-taxadienone (7) was used as the substrate for FoTO1, its complete conversion to compound 10 was also observed. The isomerization product 10 was identified as taxadienone-5α-ol by 2D-NMR, bearing the same hydroxylation pattern as T-5α-ol (5). These findings reveal a novel biosynthetic mode in which the hydroxylated product is generated stepwise from

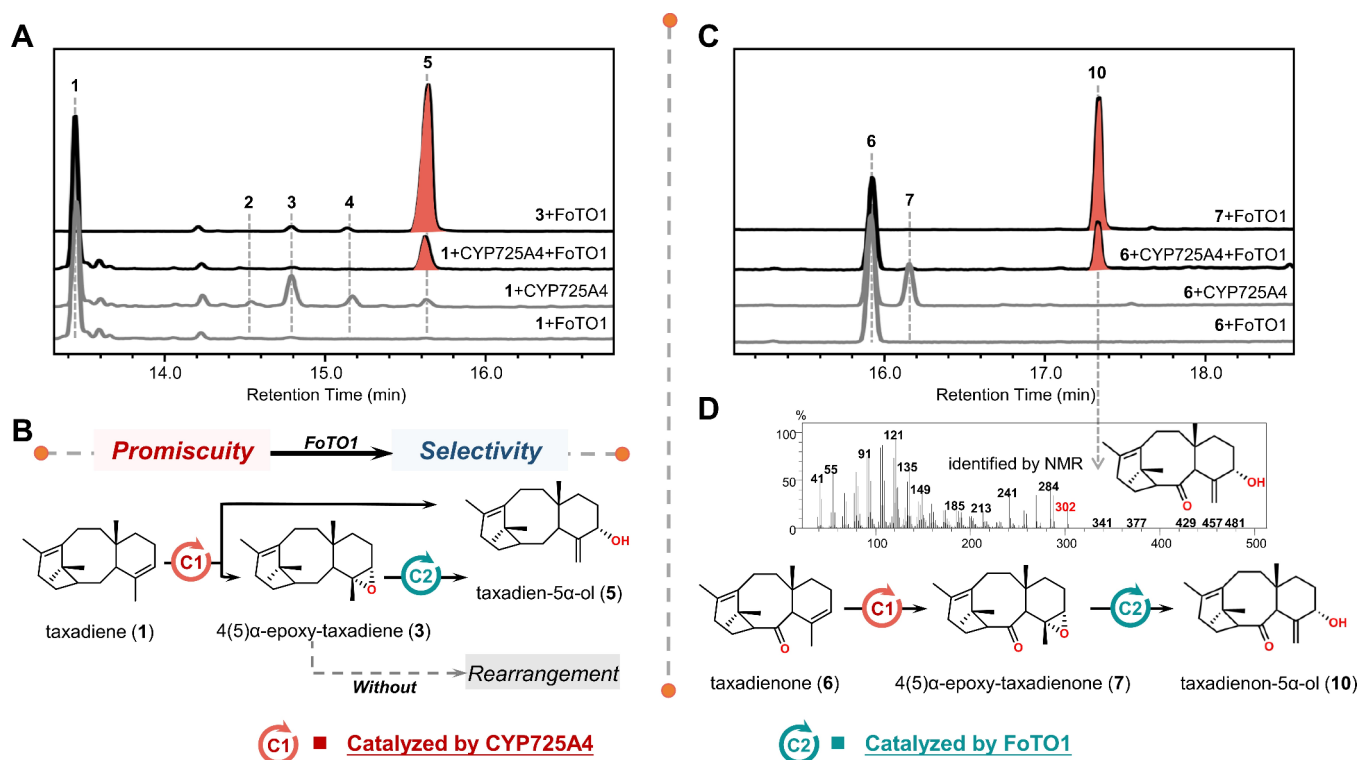


Figure 4. FoTO1-mediated taxanes isomerization. (A) GC-MS TICs of in vitro reactions with various combinations of FoTO1, CYP725A4, taxadiene (1), and 4(5)α-epoxy-taxadiene (3). (B) FoTO1-assisted selective oxidation pathway of taxadiene (1) by CYP725A4. (C) GC-MS TICs of in vitro reactions with various combinations of FoTO1, CYP725A4, taxadienone (6), and 4(5)α-epoxy-taxadienone (7). (D) Stepwise synthesis of taxadienone-5α-ol (10) by CYP725A4 and FoTO1. The isomerization product 10 was identified as taxadienone-5α-ol through NMR structural elucidation and confirmed by high-resolution EI-MS (Figures S20, S52–S57, Tables S3, and S9).

a double bond-containing substrate (Figure 4D), wherein the highly efficient FoTO1 functions as an indispensable taxane epoxide isomerase.

Semi-rational Design Enhances the Catalytic Activity of CYP725A4

The inherent interconversion of products impedes the accurate determination of CYP725A4's catalytic efficiency towards taxadiene (1), thereby hindering the analysis of mutation site effects during directed evolution. In contrast, CYP725A4 exhibits high product selectivity towards the substrate mimic taxadienone (6), forming a stable epoxidation product that enables an accurate evaluation of its catalytic efficiency. Furthermore, molecular docking confirms that taxadienone (6) adopts a binding mode highly similar to taxadiene (1) (Figure S9), indicating that taxadienone (6) can serve as a surrogate substrate for taxadiene (1) to screen for activity-enhanced CYP725A4 variants in vitro.

Many reports have been dedicated to enhancing catalytic activity by modifying amino acid residues within 5 Å of the substrate-binding pocket, yet all of these efforts remained unsuccessful for CYP725A4.^{26,28,30} In our previous study, ancestral sequence reconstruction was employed to identify two key residues (V124 and S127, corresponding to V148 and S151 herein due to an N-terminal shift caused by signal peptide truncation) located on α-helix. Positioned distally from the substrate-binding sites, these residues nevertheless significantly modulate enzymatic activity.³¹ Inspired by these findings, we performed sequence alignment between CYP725A4 and its ancestral enzymes, and the evolutionarily distinct sites located

within 5–8 Å of the substrate (L77, A127, T140, A230, and V446) were substituted with their ancestral counterparts (Figure 5A). Notably, variants V446P and A230S demonstrated improved activity towards taxadienone (6) compared to the wild-type CYP725A4 (WT). V446 is located in the heme-binding domain, while A230 resides in the FG loop adjacent to the substrate access channel. Structural analysis suggests two plausible mechanisms underlying the improved catalytic efficiency: (i) the V446P substitution in heme-binding domain may alter the heme conformation to shorten the interaction distance with the substrate, consequently impacting catalytic reactivity;⁶² (ii) the A230S mutation in the FG loop facilitates substrate access or product release through flexible gating motions,^{63–65} with the wild-type residue potentially imposing structural constraints at the channel entrance that limit activity.⁶⁶

To further optimize the heme-binding conformation and enhance catalytic efficiency, five low-conserved residues within 4 Å of heme were replaced with amino acids of varying steric bulk (Figures 5B and S10). Among the 15 constructed variants, S451T and V446L exhibited a significant increase in relative activity compared to WT. We next sought to enhance activity by engineering the flexible FG loop. In the paclitaxel biosynthetic pathway, sequential oxidative modifications of taxanes involve P450 enzymes that act on substrates with increasing structural complexity. We thus hypothesize that different taxane-hydroxylating P450s may have evolved distinct FG loop architectures to accommodate these bulky substrates. We then aligned sequences of seven characterized P450s^{14,67–71} in the

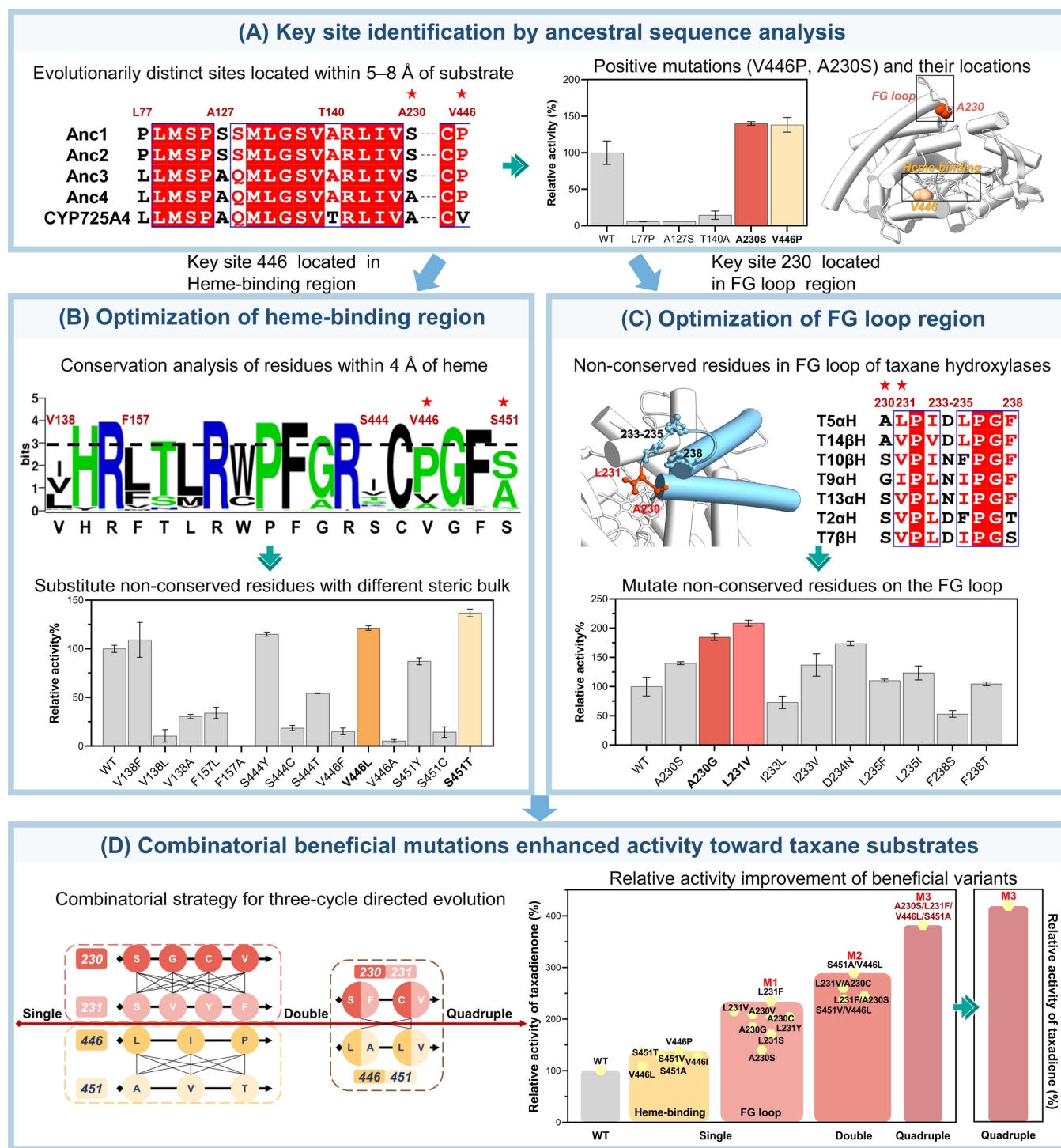


Figure 5. Workflow for the directed evolution of CYP725A4. (A) Rapid identification of key sites within 5–8 Å of the substrate through ancestral sequence analysis. The significantly different residues between CYP725A4 and its ancestral enzymes were substituted with the corresponding ancestral amino acids. (B) Scanning of non-conserved sites within the 4 Å heme-binding domain. Nonpolar residues were substituted with F, L, or A to introduce different steric bulk, while polar residues were replaced with Y, C, or T to achieve similar variation. (C) Scanning of non-conserved residues in the FG loop. Non-conserved sites were substituted with the corresponding residues from other cytochrome P450 hydroxylases involved in the paclitaxel biosynthesis pathway. (D) The oxygenation activity of beneficial single-point and combinatorial variants relative to CYP725A4 WT. Relative activity of taxadienone (6) was quantified based on its sole oxygenated product 4(5) α -epoxy-taxadienone (7), while that of taxadiene (1) was calculated from all oxygenated products, including iso-OCT (2), 4(5) α -epoxy-taxadiene (3), OCT (4), and T-5 α -ol (5).

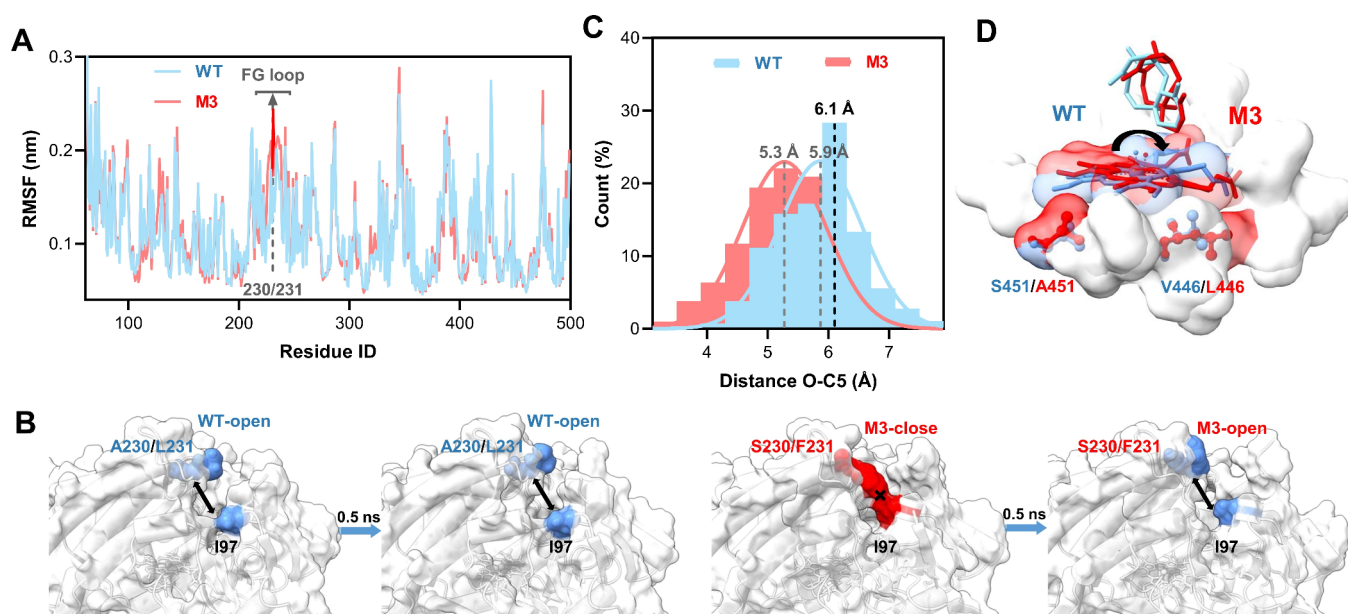


Figure 6. Molecular mechanism of enhanced catalytic activity in M3 revealed through comparative MD simulations with WT. (A) Average root-mean-square fluctuation (RMSF) values per residue for WT and M3 during MD simulations. (B) Representative sequential snapshots (every 0.5 ns) of the substrate channel entrance in WT and M3. Residues 231 and 97 are color-coded indicate conformational states (red: closed conformation; blue: open conformation). (C) Distribution of distances between the oxygen atom of Cpd I and the C5 atom of taxadiene (1) in WT and M3 during total 400 ns MD simulations. Gaussian least-squares fitted curves and mean values are shown. (D) Superimposed structures of the heme-binding domain in WT and M3. Black arrows indicate the direction of conformational shift induced by altered steric hindrance at mutation sites. Mutated residues are shown as ball-and-stick models (blue: WT; red: M3).

paclitaxel biosynthetic pathway, and selected six non-conserved sites for mutagenesis by introducing residues from homologous P450s (Figure 5C). The majority of the ten variants showed enhanced relative activity, with L231V exhibiting a 2.1-fold increase over WT, validating the effectiveness of this engineering strategy.

Site saturation mutagenesis was conducted at key positions (the positions of two beneficial mutants from each of the two scanned regions, heme-binding region: 446 and 451, FG loop region: 230 and 231) to identify optimal substitutions (Figure S11). Positive variants in the FG loop conferred significantly higher activity improvements than those in the heme-binding domain. L231F (M1) exhibited the highest relative activity among all single-point variants. Positive single mutations for each region were then combinatorially assembled to generate double-site variants (Figure S12), and favorable double variants were further combined to construct quadruple-site variants (Figure S13). Through three rounds of directed evolution, the best-performing variant A230S/L231F/V446L/S451A (M3) achieved a 381% increase in relative activity toward the substrate mimic taxadienone (6), and also showed a 419% improvement for the natural substrate taxadiene (1) (Figure 5D).

Molecular Mechanism of Increased Catalytic Activity

Molecular dynamics (MD) simulations were conducted for WT and M3 to explore the structural basis underlying the improved activity of CYP725A4. The root-mean-square deviation (RMSD) quantifies the dynamic fluctuations of the protein backbone throughout the MD process (Figure S14). Two independent simulations showed comparable structural stability

between WT and M3. Subsequently, structural flexibility was probed through analyzing the averaged values of root-mean-square fluctuation (RMSF) (Figure 6A). M3 exhibited significantly elevated RMSF values specifically in the FG loop region compared to WT, suggesting that the A230S/L231F mutation in this region may enhance the loop flexibility. Further analysis of the stabilized MD trajectories suggested distinct conformational dynamics between WT and M3 variants (Figure 6B). While WT remained an open substrate entrance throughout, M3 sampled both open and closed conformational states. This conformational behavior may be attributed to the bulky phenylalanine side chain and more flexible structure introduced by the A230S/L231F mutation, which may function as a substrate gate and undergo frequent conformational transitions between open and closed states. Consistent with studies of other P450 enzymes,^{72,73} enhanced flexibility in the FG loop increases substrate cavity dynamics and adaptability, potentially facilitating substrate binding and catalytic efficiency.

To provide mechanistic insights into the enhanced catalytic efficiency resulting from the heme-binding region mutation, the distance between the oxygen atom of Compound I (Cpd I) and the C5 atom of taxadiene (1) (distance O–C5) was measured during MD simulations, which is associated with the catalytic activity of P450s (Figure 6C). The most frequent attack distance O–C5 in M3 (5.3 Å) is shorter than that in WT (5.9 Å). Moreover, M3 exhibited a higher occurrence of catalytically favorable conformations (distance O–C5 ≤ 5 Å, 11.4% in WT, 37.5% in M3), which is consistent with the experimentally observed enhancement in oxygenation activity of M3 compared to the WT. Structural analysis suggested that the V446L/S451A mutation is associated with heme tilting by

modulating steric hindrance, increasing at V446L while decreasing at S451A. This repositions the oxygen atom of Cpd I closer to the substrate's C5 atom in M3, thereby potentially facilitating the P450-catalyzed oxygenation (Figure 6D).

Heterologous Co-expression of the Optimal Variant of CYP725A4 and FoTO1 in *E. coli* Enables High-Yield Production of T-5 α -ol

To assess the catalytic efficiency of the optimal CYP725A4 variant in producing the key paclitaxel intermediate T-5 α -ol (5) in vivo, an engineered taxadiene-producing *E. coli* strain was constructed according to our previous work (Figure S15).³² Two derived strains, S1 and S2, were generated to express either WT or the variant M3 of CYP725A4, along with a cytochrome P450 reductase (CPR) to supply electrons for P450 catalysis. In the cellular environment during fermentation of strain S1, the rearrangement of 4(5) α -epoxy-taxadiene (3) was also observed; and similarly, the product proportion of T-5 α -ol (5) did not change significantly (Figure S16). After 72 h shake-flask fermentations, strain S2 with M3 increased the titer of T-5 α -ol (5) from 1.6 to 3.6 mg/L, and the total oxygenated products rose to 53.8 mg/L, though the relative proportion of T-5 α -ol (5) remained largely unchanged (Figure 7A). To address the formation of dead-end products in taxadiene (1) oxygenation catalyzed by CYP725A4, strains S3 and S4 were constructed by heterologously co-expressing FoTO1 in strain S1 and S2, respectively. In strain S3, no detectable levels of iso-OCT (2), 4(5) α -epoxy-taxadiene (3), or OCT (4) were observed (Figure S17), and T-5 α -ol (5) titer reached 32.8 mg/L. Strain S4, co-expressing the optimal variant of CYP725A4 and FoTO1, achieved a maximum titer of 52.6 mg/L for T-5 α -ol (5). These results demonstrate that coordinated engineering of the key enzyme CYP725A4 and heterologous co-expression of FoTO1 are essential for enhancing the biosynthesis of paclitaxel intermediates.

The highest titer of T-5 α -ol (5) was achieved through scaled-up fermentation of strain S4 in a 5 L bioreactor. At 40 h of fermentation, T-5 α -ol (5) reached a peak titer of 109.1 mg/L

(Figure 7B), with no detectable formation of by-products. Only trace amounts of the dioxygenated product 9 of T-5 α -ol (5) could be detected in the late stage of fermentation (Figure S18). This result constitutes the highest reported titer for de novo microbial synthesis of T-5 α -ol (5). Notably, product accumulation was closely correlated with cell growth, and the rapid production phase required an adequate supply of precursors (Figure S19), indicating that the current production system and conditions remain suboptimal and warrant further investigation.⁷⁴

DISCUSSION

The recent discovery of key genes in several specific metabolic steps, particularly FoTO1,¹² C4 β -C20 epoxidase,¹³ taxane oxetanase,¹⁴ has accelerated the development of paclitaxel biosynthesis. However, the complex catalytic mechanisms of the functional enzymes involved in paclitaxel biosynthesis remain unclear, restricting further yield improvements through protein engineering. This issue is especially pronounced with CYP725A4, the first enzyme of taxadiene (1) functionalization, as its catalytic promiscuity severely hinders the downstream synthesis.^{17,21,75}

Through oxygenation of the structural analogs of taxadiene (1), we identified taxadienone (6) as a substrate mimic selectively epoxidized at the C4=C5 double bond by CYP725A4. Prompted by this finding, we isolated and characterized the major taxadiene (1) oxygenation product—a long-observed yet uncharacterized compound—as 4(5) α -epoxy-taxadiene (3). This identification is essential for understanding the catalytic promiscuity of CYP725A4, which appears to arise from non-selective epoxidation and hydroxylation activities, followed by epoxide rearrangement and subsequent oxygenation of derived products. Distinct from the previously proposed mechanisms (Figure 1), our findings suggest that T-5 α -ol (5) is generated via P450-mediated hydroxylation that occurs concurrently with epoxidation, rather than through rearrangement of 4(5) α -epoxy-taxadiene (3).³⁰ Additionally, the byproducts OCT (4) and iso-OCT (2) arise from the spontaneous

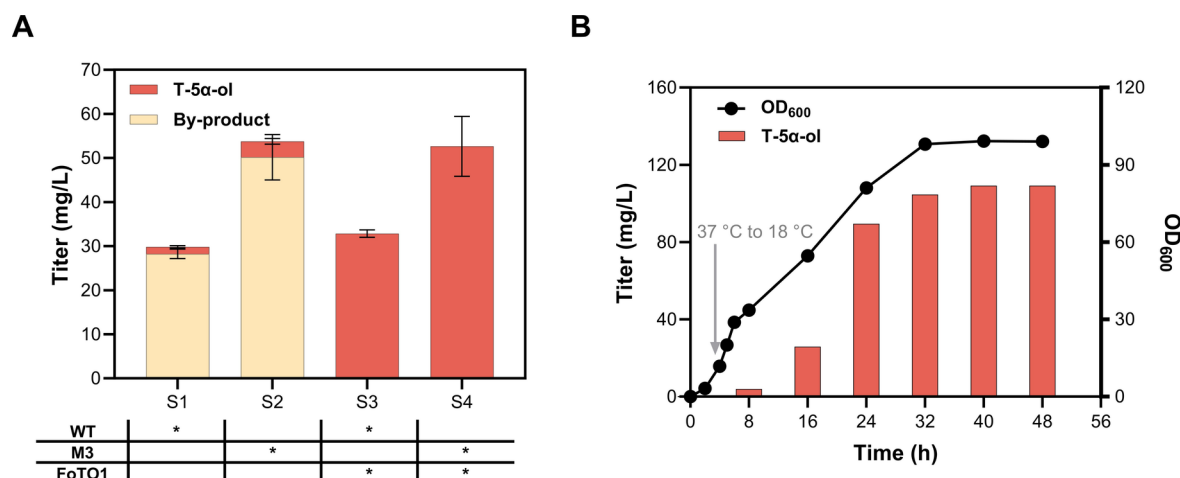


Figure 7. De novo synthesis of T-5 α -ol (5) in engineered *E. coli* with CYP725A4 (WT or M3) and FoTO1. (A) Titer of T-5 α -ol (5) in 50 mL shake-flask culture. Different combinations of wild-type or M3 mutant CYP725A4 and FoTO1 were expressed in the T-5 α -ol (5)-producing strain. The table below indicates the CYP725A4/FoTO1 combination used for each strain. Data represent the mean $\pm$ SD of three independent replicates. (B) Fed-batch culture of strain S4 in a 5 L bioreactor. When OD₆₀₀ reached 15–20, the temperature was lowered to 18 °C for the production of T-5 α -ol (5).

rearrangement of 4(5) α -epoxy-taxadiene (3), not from CYP725A4-catalyzed radical rebound or protonation of a zwitterionic intermediate.^{26,30} Regarding the successive epoxidation events catalyzed by CYP725A4, only 4 β ,19-ether-taxadiene-5 α -ol (8) was observed, while the formation of taxogenenic oxetane reported in *S. cerevisiae* and *N. benthamiana* systems⁵⁹ was not detected in this *E. coli*-based in vitro catalytic system. This suggests that the formation of the oxetane ring may require the involvement of additional oxidase enzymes.^{13,14,76,77} Moreover, the structural complexity of the dioxygenated products (8 and 9) likely stems from site-selective oxygenation and subsequent rearrangements by CYP725A4, and further work will provide new insights into the P450-mediated formation of taxane structural diversity.⁷⁸

The pronounced difference in catalytic selectivity between taxadienone (6) and taxadiene (1) raises an interesting question for future investigation: how does substrate structure dictate catalytic selectivity? Our preliminary docking analysis indicated that steric hindrance imposed by the ketone group positions both the C5 and C20 atoms of taxadienone (6) farther from the heme center than those in taxadiene (1), which may impede the oxygen rebound step required for hydroxylation and thereby underlie its selective epoxidation.³⁰ Recently, using diverse substrates as functional probes has elucidated how substrate structure governs the hydroxylation versus epoxidation selectivity in a 2-oxoglutarate-dependent oxidase.^{79,80} Furthermore, FoTO1 promotes the formation of the desired product in a stepwise manner through isomerization, thereby significantly enhancing the metabolic flux. More importantly, this isomerization of an epoxide to an allylic alcohol represents a novel reaction type in biocatalysis,^{81,82} further illustrating the diversity of natural product biosynthetic processes.

Building upon the catalytic selectivity observed with taxadienone (6), the analytical challenges associated with the complex mixture of products generated from promiscuous taxadiene (1) oxygenation are minimized, thereby enabling efficient screening for CYP725A4 variants with enhanced catalytic efficiency. The FG loop and the heme-binding domain—regions not directly involved in substrate binding—were targeted for improving the catalytic activity of CYP725A4. This approach reflects a broader trend in P450 engineering, where loop optimization has proven effective in boosting enzymatic activity, consistent with findings in other P450 systems.^{83–85}

Ultimately, the plant-derived protein FoTO1 demonstrated excellent compatibility in the heterologous expression system. This allowed efficient co-expression of FoTO1 and the CYP725A4 variant M3 in engineered *E. coli*, elevating T-5 α -ol (5) production to 109.1 mg/L. Collectively, these results lay a foundation for constructing downstream heterologous biosynthetic pathways toward paclitaxel, and elucidating the catalytic mechanisms of individual enzymes along with their proper integration into a functional metabolic network will be key to further enhancing production.^{14,16,86,87}

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.6c04534>.

Experimental details, NMR spectra for compounds 1–10, GC-MS spectra for all compounds, and supporting figures (PDF)

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<https://pubs.acs.org/doi/10.1021/acscatal.6c04534>

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the Shanghai Commission of Science and Technology (No. 23HC1400200), the National Natural Science Foundation of China (Nos. U24A20484 and 22478116), and the Shanghai Municipal Science and Technology Major Project. We are thankful to Romas Kazlauskas (College of Biological Sciences, University of Minnesota, Minnesota, US) for helpful discussions.

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