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Mechanistic Insights into Taxadiene Epoxidation by Taxadiene-5α-Hydroxylase

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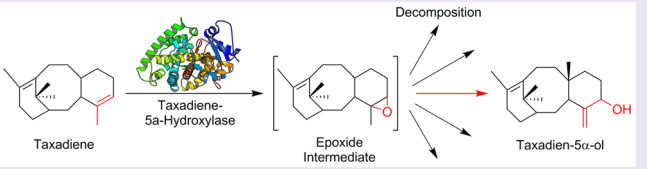
Supporting Information

ABSTRACT: The anticancer molecule taxol (Paclitaxel) stands as one of the most medically and economically important natural products. However, despite decades of extensive study, its biosynthesis remains poorly understood. Unpredictable behavior of the first oxygenation enzyme, taxadiene-5α-hydroxylase, which produces a range of undesired products, currently stands as a key bottleneck to improved taxol production. We herein present chemical and biological evidence of an unreported epoxidase activity of taxadiene-5α-hydroxylase that puts into question the previously proposed radical-rebound mechanism. We demonstrate that the poor selectivity of taxadiene-5α-hydroxylase arises from nonselective degradation of an epoxide intermediate produced via a selective oxidation step, rather than from promiscuous oxidation, as previously proposed. We support these conclusions by demonstrating variable enzyme behavior in differing hosts and conditions, similarity of products and product ratios generated from chemical epoxidation, and taxadiene-5α-hydroxylase, and differing enzymatic activity on alternative taxadiene isomers. Additionally, we use directed mutagenesis to describe the oxidizing species of the P450, demonstrate that further *in vivo* functionalization of oxidized taxadiene is unable to improve selectivity of the oxidation, and show that multiple products are produced in the *Taxus cuspidata* and are not simply an artifact of heterologous expression. Our results highlight an important, and previously unknown, obstacle to improved taxol production. We further offer insights to overcome the challenges posed by an epoxide-mediated reaction, which sets the basis for further engineering of taxol biosynthesis.



The plant natural product taxol (Paclitaxel, Abraxane) has attracted the interest of biologists and chemists for decades due to its potent anticancer properties as well as its complex, highly functionalized structure.[1−5] Current production relies on the native capacity of plants, through harvesting needles or culturing plant cell culture. Natively, plant cells produce low amounts of taxol over long culture times.[6] Thus, engineering of native plant metabolism or the heterologous production of taxol in microbial hosts could substantially improve taxol production.[7]

The biosynthesis of taxol (outlined in Figure S1) begins with the cyclization of the isoprenoid precursor geranylgeranyl pyrophosphate (GGPP) to form taxa-4(5),11(12)-diene (taxadiene) by taxadiene synthase (TXS).[8] Next, taxadiene is hydroxylated at the 5α position by the cytochrome P450 taxadien-5α-hydroxylase (CYP725A4, here referred to as 5αCYP).[9,10] This product, taxa-4(20),11(12)-dien-5α-ol (T5OH), has been shown to undergo further hydroxylation at the 13α, 10β, and 14α positions, with hydroxylation at the 13α or 10β positions capable of biotransformation into taxol in the native host.[11−13]

While heterologous overproduction of early paclitaxel precursors in heterologous hosts such as *Saccharomyces cerevisiae* and *Escherichia coli* could provide access to valuable taxol precursors, these efforts have shown limited success, despite using only previously characterized enzymes.[1,3,14] This has been largely due to the unpredictable behavior of the first enzymatic step catalyzed by 5αCYP. Large amounts of the first cyclization product (taxadiene) can be produced heterologously, and taxadiene can be converted to oxygenated compounds at relatively high yield.[3] A majority (85% to 95%) of these oxygenated compounds, however, are undesired byproducts, while the desired taxol precursor (T5OH) is produced with only 5% to 15% selectivity in many reconstituted systems.[1,3] Thus, selectivity of the oxygenation step by 5αCYP stands as a key obstacle to heterologous taxol production.

Cytochrome P450s, such as 5αCYP, are catalytically powerful enzymes capable of activating alkyl C−H or C=C bonds, typically via hydroxylation or epoxidation. The hydroxylation catalyzed by 5αCYP has been proposed to occur via a "radical rebound" mechanism[10] (Figure S2). In line with many P450s,[15] radical rebound begins with the abstraction of a hydrogen radical from the C20 position of taxadiene by an oxoiron(IV) species cofactor (Fe^{IV}=O; referred to as Cpd I[16]), forming an allylic radical intermediate (Figure 1A, #2). The heme-bound hydroxyl group then "rebounds" to the 5α position, resulting in both a hydroxylation and an allylic rearrangement, producing T5OH. This initial finding was supported by multiple lines of

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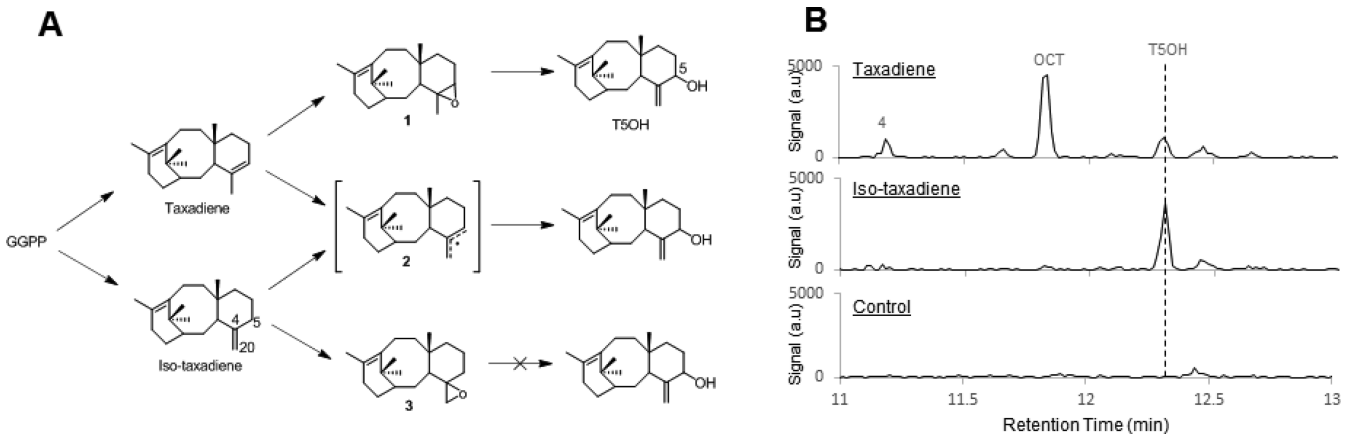

Figure 1. Possible reaction cascades for taxadiene isomers. (A) The catalytic route from the linear precursor GGPP to T5OH begins with cyclization to taxadiene by TXS, with TXS displaying ~95% selectivity for taxadiene and ~5% for iso-taxadiene. Taxadiene is then acted upon by 5αCYP and can then feasibly proceed to T5OH via either an epoxide intermediate (1) or a radical intermediate (2) through radical rebound. Iso-taxadiene may also undergo either radical rebound (2) or epoxidation (3). However, as iso-taxadiene cannot yield T5OH though epoxidation (3) without complex rearrangement, it must logically proceed to T5OH via the proposed radical intermediate. (B) Formation of oxygenated products catalyzed by engineered *E. coli* expressing 5αCYP when supplemented with taxadiene, iso-taxadiene, or no substrate, as confirmed by GCMS analysis.

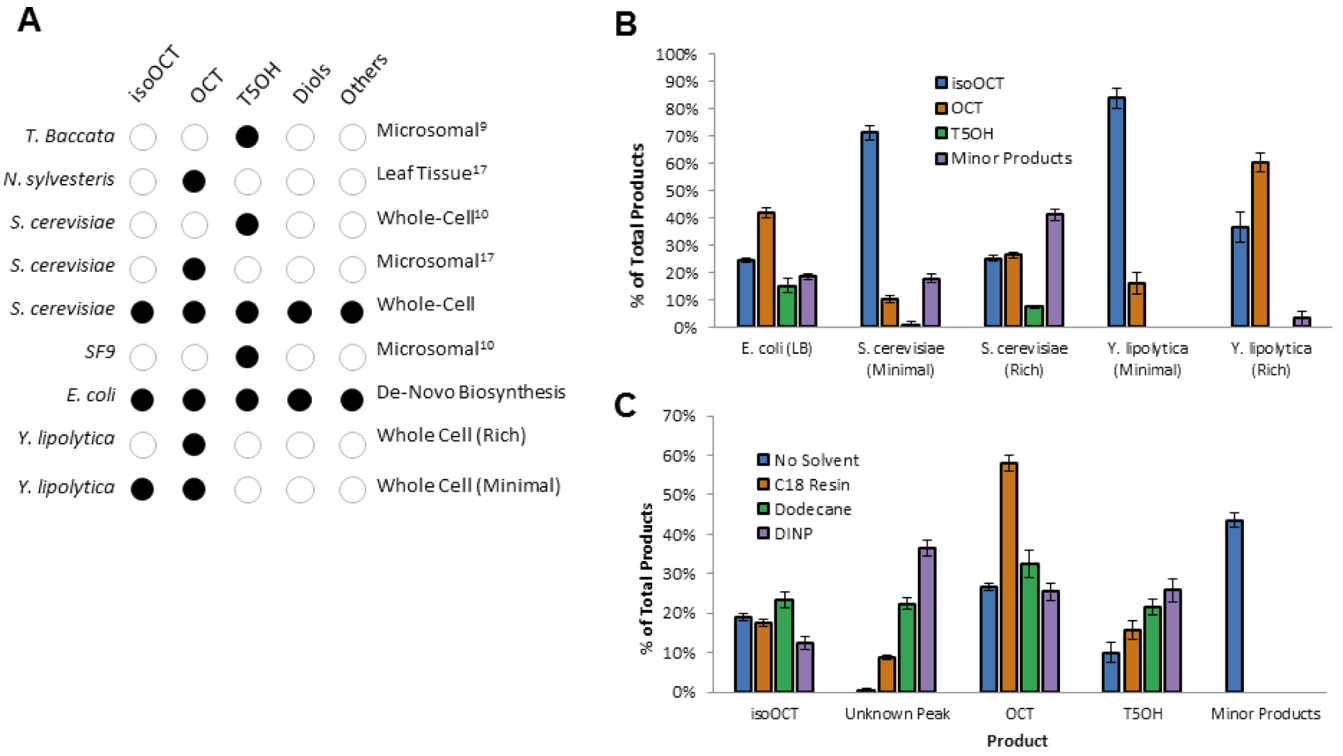

Figure 2. Selectivity of taxadiene-5α-hydroxylase in varying hosts and systems. (A) Summary of literature and experimental results on 5αCYP selectivity in various systems. Dark circles indicate products observed when exogenous taxadiene is supplied. Of note is that an identical *S. cerevisiae* strain (WAT11), when assayed using microsomes[17] or whole cells[10] produced differing selectivity profiles, indicating assay conditions may influence selectivity. (B) Selectivity for oxygenated products obtained from feeding of taxadiene to whole-cells expressing 5αCYP in different systems, normalized to total production. Strains depicted are TaxE9, TaxS1, and TaxY1; rich media denotes YPD and minimal media denotes YNB. Three primary products are shown; minor products, due to their low abundance, are grouped. These minor compounds include only observed compounds with a parent mass of 288 m/z, indicating monohydroxylation. (C) Variations observed from *E. coli* cultured in the presence of extractive fermentation agents. Significant shifts in selectivity are shown, with an additional product (unknown A, parent mass 288 m/z), which is observed only in extractive fermentation regimes displayed as well.

evidence[9,10] but has been challenged by subsequent studies, in which another compound, 5(12)-oxa-3(11)-cyclotaxane (OCT), was identified as the primary enzymatic product.[1,17] It has been proposed that these differences arise from a natively broad product profile resulting from promiscuous oxidation of taxadiene.[1,18] However, as consistent findings for the 5αCYP selectivity have not been achieved, we propose that the broad product profile of taxadiene oxidation points to a nonenzymatic descriptor of the reaction promiscuity.

In this study, we present data in support of taxadiene epoxidation within 5αCYP and illustrate how this may resolve conflicting reports of enzyme selectivity. We further demon-

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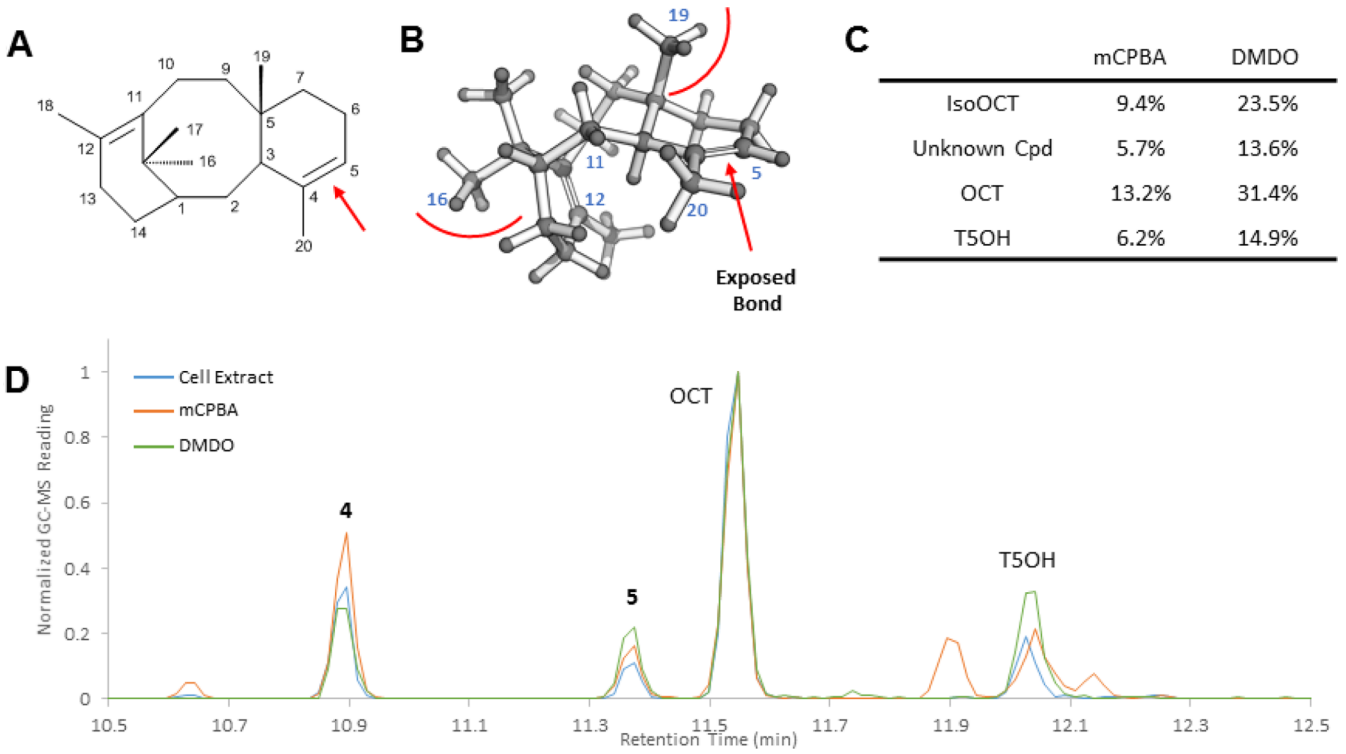

	mCPBA	DMDO
IsoOCT	9.4%	23.5%
Unknown Cpd	5.7%	13.6%
OCT	13.2%	31.4%
T5OH	6.2%	14.9%

Figure 3. Chemical synthesis of putative epoxide intermediate. (A) Taxadiene with predicted reaction site of chemical epoxidation indicated by an arrow. (B) Three-dimensional representation of taxadiene displaying steric hindrance arising from the C16 methyl group and concave face of the molecule preventing nucleophilic attacks at the C11−C12 double bond and from the C19 methyl group preventing attack at 5β position. (C) Yields obtained from optimized mCPBA and DMDO reaction conditions, showing nearly 80% turnover to oxygenated products from the DMDO reaction. (D) GCMS traces of reaction products from DMDO (green), mCPBA (orange), compared to extracts from *E. coli* (TaxE12) grown in the presence of dodecane (blue); normalized to maximum observed TIC. *E. coli* in the presence of solvent was chosen for comparison due to its proposed ability to sequester unstable intermediates, allowing approximation of the uninfluenced selectivity of the enzyme.

strate that the broad product profile may arise from nonselective degradation of the proposed epoxide intermediate. Although the epoxide intermediate itself proved too unstable for structural characterization, indirect evidence presented here supports its existence as a product of taxadiene oxidation by 5αCYP. This stands in contrast to the previously proposed radical rebound mechanism in which a broad product profile of taxadiene oxidation arises from poor 5αCYP selectivity.[18]

We first present data that are incompatible with radical rebound including observed enzyme product profile differences and product identities. We next establish support for a taxadiene epoxidation mechanism by demonstrating that chemical epoxidation reactions produce a similar suite of products to that obtained *in vivo*. We also demonstrate that 5αCYP produces a single oxidation product from a taxadiene regioisomer and a mixture of products from taxadiene itself, whereby we propose that the native product profile of 5αCYP results from degradation of a putative taxadiene epoxide rather than poor enzyme selectivity. The establishment of this epoxide intermediate reveals a significant, and previously unknown, obstacle to high-level taxol biosynthesis in both native and heterologous systems.

RESULTS AND DISCUSSION

Characterization of Enzyme Selectivity under Varying Conditions. To understand factors influencing the selectivity of 5αCYP, we began by comparing conflicting reports on the product profile obtained from 5αCYP (Figure 2A). These reports suggest assay conditions play a key role in observed selectivity, and thus we set out to probe the effect systematically, with host choice selected as an initial point of investigation. 5αCYP and its native redox partner (CPR) were coexpressed in three common lab strains, *E. coli* (TaxE9), *S. cerevisiae* (TaxS1), and *Yarrowia lipolytica* (TaxY1), and cultured in their corresponding rich and minimal media. Taxadiene was supplemented exogenously, and product distribution was determined via GCMS (Figure 2B). As depicted in Figure 2B, multiple products were observed, including three major and four minor monohydroxylated compounds (parent ion 288 m/z), as well as low levels of compounds with mass spectra indicating dihydroxylation (parent ion 304 m/z, not depicted). As shown, different expression hosts and media conditions both had a dramatic impact on selectivity of 5αCYP. There is little precedent for heterologous enzymes to exhibit differential selectivity of this nature; thus we hypothesized that changes in selectivity were not attributable to changes in the enzyme itself, but rather to the presence of a reactive intermediate such as an epoxide. We propose that the transformation of taxadiene to T5OH would require multiple steps, between which host metabolism or intracellular conditions may intervene to alter product distribution, giving rise to the observed differences in enzyme selectivity.

To further probe the existence of a multistep pathway, we examined the effect that extractive fermentation, or the addition of an organic solvent to the fermentation medium, would have on product distribution. In multistep pathways or situations in which enzymes catalyze sequential reactions, addition of an

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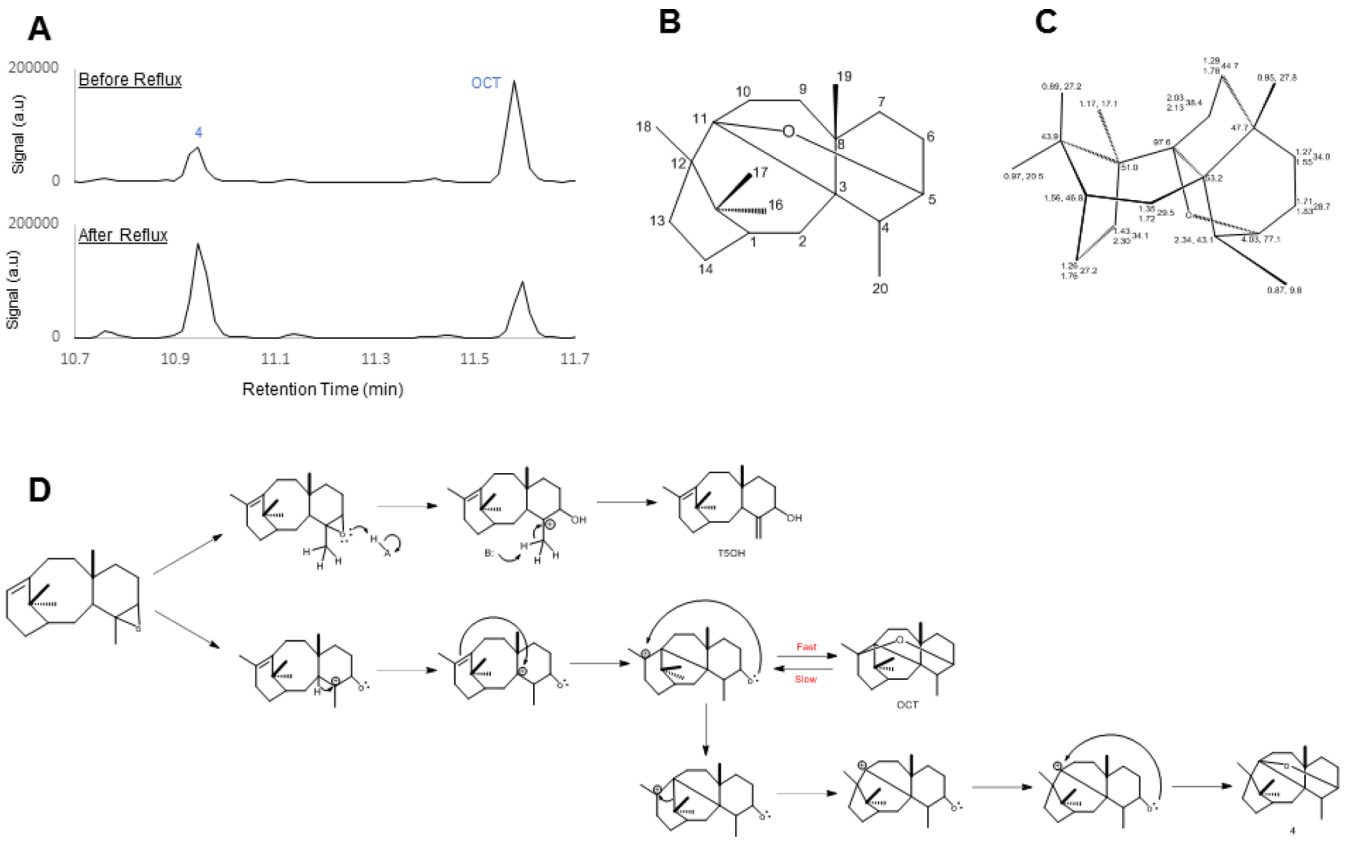

Figure 4. Elucidated structure of OCT and possible biosynthetic origin. (A) GCMS chromatogram displaying conversion of OCT to **4** upon reflux in acetonitrile. (B) Stick representation of the newly elucidated compound **4** structure. As can be seen, the center ring has expanded from an eight-membered to a nine-membered ring, and a C−C bridge between C3 and C11 is present, as in OCT.[17] (C) Three-dimensional representation with observed carbon and hydrogen chemical shifts are shown. (D) Proposed cascade to known products from epoxide intermediate **1**, showing feasibility of formation of OCT, T5OH, and **4**. The pathway depicted: we depict a route from which **4** can be generated both directly from the epoxide intermediate, as well as from OCT, in line with existing data.

organic solvent has been shown to affect product profile, presumably by altering the concentrations of reactants, products, and pathway intermediates.[19,20] We thus cultured *E. coli* cells containing the entire biosynthetic pathway (TaxE12) in the presence of dodecane, diisononyl phthalate (DINP), or a solid C18 chromatography resin. Each extractive method displayed a unique product distribution (Figure 2C). Although one may feasibly propose dodecane and DINP influence selectivity via direct interaction with the 5αCYP enzyme, the ability of the solid C18 resin to alter selectivity argues against this, since the particles (40−60 μm) are too large to enter the cell or directly interact with the enzyme itself. This result, and the one above, indicates the presence of stable intermediates. This is again irreconcilable with radical rebound, in which the radical form is a short-lived intermediate present only within the active site of the enzyme.[10]

We thus hypothesized that hydroxylation of taxadiene by 5αCYP occurs via an epoxide intermediate (Figure 1A, #1), as is common in many P450s. In this scenario, the pathway from taxadiene consists of two distinct steps: epoxidation catalyzed by 5αCYP followed by degradation to the observed products. Under this scheme, the variation in selectivity between hosts can be explained by adventitious enzyme activity on epoxide **1** or intracellular host conditions, and variations observed in extractive fermentation can be attributed to properties of the solvent in which the epoxide is sequestered.

Chemical Epoxidation Reproduces 5αCYP Product Profiles. In contrast to a radical-mediated taxadiene oxidation, extractive fermentation supported the presence of a stable intermediate. We set out next to confirm that the product distribution observed in *E. coli* can result from degradation of a putative epoxide intermediate. Thus, we sought to chemically synthesize taxen- C4(C5) monoepoxide **1** for feeding to *E. coli* lacking the taxadiene biosynthetic pathway and 5αCYP (TaxE0). This strategy would remove any influence of 5αCYP on the oxidized taxadiene pool and test whether the epoxide is capable of degradation to the observed products *in vivo*.

Conformational analysis of taxadiene depicts a steric preference for epoxidation on the internal face of the C4−C5 olefin (Figure 3A,B). We therefore anticipated that mono-epoxidation of taxadiene with a mild, nonselective epoxidation reagent such as dimethyldioxirane (DMDO) or meta-chloroperoxybenzoic acid (mCPBA) would yield only the monoepoxide product **1** in a regio- and stereoselective manner. However, following the reaction, GCMS analysis of the crude products revealed multiple peaks rather than a single epoxide. The retention times, mass spectra, and total ion count (TIC) ratios of these peaks matched those previously observed using *E. coli* TaxE12 in the presence of a solvent (Figure 3D). Reaction optimization, including the use of an acetone-free DMDO preparation performed at −78 °C, failed to alter selectivity or produce only the desired epoxide. We believe this

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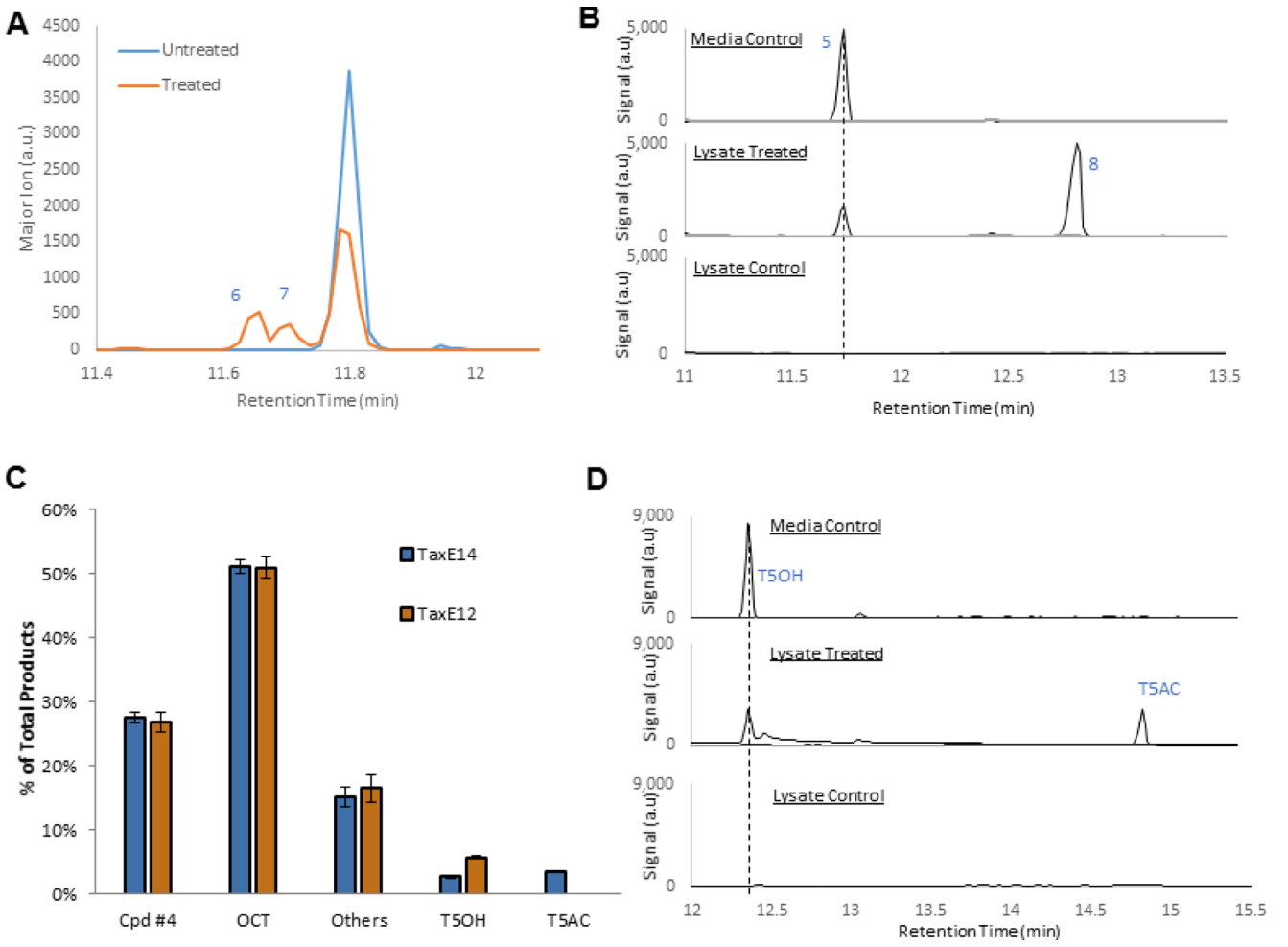

Figure 5. Further transformations of 5αCYP products. (A) GCMS chromatogram of products generated via acid hydrolysis of Cpd #5, demonstrating two new peaks, which do not coelute with any previously observed compounds. (B) GCMS chromatogram of compounds generated via incubation of #5 with *E. coli* crude lysates, showing a novel compound at RT = 12.8 min. (C) Selectivity for oxygenated taxanes in *E. coli* cells with (TaxE14) and without (TaxE12) taxadien-5α-ol O-acetyltransferase, displaying no significant shift in overall selectivity for compounds other than T5OH with expression of the downstream enzyme. (D) GCMS chromatogram displaying conversion of T5OH to Taxa-4(20),11-dien-5alpha-yl acetate (#9).

most likely indicates epoxide degradation upon warming, impeding our ability to isolate and directly characterize the epoxide. Furthermore, a combination of poor stability and structural complexity of taxadiene and the observed reaction products impedes the absolute characterization of the epoxide **1** via direct methods such as *in situ* low-temperature NMR during the reaction.

The observation that these biologically derived isomerization products are also products of chemical epoxidation of taxadiene implicates **1** as a common intermediate for both the chemical and biological reactions. This further suggests that the biosynthetic products result from degradation of the epoxide and not from multiple reaction pathways of a promiscuous enzyme. Furthermore, these experiments highlight the instability of the cup-shaped epoxide molecule, which one might surmise promotes nucleophilic attack of the C12−C13 olefin into the newly formed epoxide, thus preventing its characterization.

Characterization of Epoxidation Products. We next aimed to characterize the four major reaction products observed in both the biological and the chemical reactions above. Two of the observed products match GCMS retention times and mass spectra to previously reported taxadiene derivatives, T5OH and OCT (retention time = 12.3 and 11.8 min, respectively;[1,9,17] Figure S3). The two remaining compounds could be isolated individually from the DMDO and mCPBA reactions, as well as biological extracts, via HPLC (compounds 4 and 5; Figure 3D), confirming that they are, indeed, stable reaction byproducts and not simply artifacts of thermal degradation during GCMS analysis.

We aimed to purify **4** from the crude epoxidation reaction material; however, fractionation via HPLC yielded two isolates containing **4**. One of these isolates contained **4** in pure form, while another fraction contained it as a mixture with OCT. As the quantity of **4** observed in the pure fraction was less than 10% of the total **4** observed in the mixture with OCT, we hypothesized that **4** could also be generated from degradation of OCT. To support this, we warmed the HPLC-isolated (semipure) OCT to reflux in acetonitrile (t = 2 h). Following reflux, the concentration of **4** was observed to have increased while the quantity of OCT had decreased, indicating that **4** could be generated, at least in part, through OCT decomposition (Figure 4A). Next we isolated compound **4** and solved its structure with NMR spectroscopy (Table S9).

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Similar to OCT, compound **4** exhibits a polycyclic carbon framework and a cup-forming tetrahydrofuran ring (Figure 4B,C). This structure allows one to propose a feasible mechanism by which compound **4** may be derived from OCT or directly from taxadiene, as illustrated in Figure 4D.

The formation of the OCT and **4** in the biological reaction is difficult to reconcile with the radical rebound mechanism. Previous reports predicted that a covalently bound heme-taxane carbocation intermediate rearranges to form OCT, yet this mechanism is without precedent.[17] In contrast, epoxidations catalyzed by P450s are common, while epoxidation followed by concomitant ether ring expansions and carbon−carbon bond formation, such as those proposed here for OCT and **4**, are well represented in terpenoid biosynthesis.[21] Additionally, a comparison of **4** to other known taxadiene oxidation products allows the proposal of a common mechanism by which all known products may be obtained from epoxide **1** (Figure 4D).

Interestingly, the other unknown compound **5** was observed only under extractive fermentation regimes and from chemical transformations. Although instability precluded its isolation and characterization, we aimed to investigate the possibility that **5** either acts as a precursor to other observed products or is epoxide **1**, by studying its decomposition. Following HPLC purification and incubation of **5** in the presence of acid, two previously undetected compounds, rather than any of the previously observed products, were detected, leading us to believe that **5** was not epoxide **1** (Figure 5A and Figure S4). To rule out differences arising from biological systems, we then assayed its transformation by crude lysates of *E. coli*. Crude lysates were able to rapidly convert **5** to a compound with a parent ion of 290 m/z (Figure 5B, Figure S4D), dissimilar to all other observed products, but again insufficient quantity could be obtained for characterization. Feeding **5** to whole cells of various species showed that the appearance of this compound varied between host organisms (Figure S5). Taken together, these results indicate that extractive fermentation leads to the accumulation of compound due to its effective removal from the cell, thus preventing its degradation by host metabolism. Furthermore, these results illustrate that this compound is not likely a precursor to other observed products.

Alternative Taxadiene Isomer Utilization Supports Epoxidation Route. An alternative taxadiene isomer, taxa-4(20),11(12)-diene (iso-taxadiene, Figure 1A), is capable of transformation into T5OH by 5αCYP[10] but cannot feasibly do so via the proposed epoxidation mechanism. To reconcile our assertion that taxadiene proceeds via epoxidation with the fact that iso-taxadiene cannot reasonably yield T5OH via this route, we proposed that the two isomers undergo distinct oxidation processes catalyzed by 5αCYP. In this regime, taxadiene undergoes epoxidation while iso-taxadiene undergoes hydroxylation via the aforementioned radical rebound mechanism (Figure 1A). Such substrate-controlled preference for hydroxylation versus epoxidation has been proposed in other systems.[22,23]

To test this hypothesis, we performed substrate feeding of both taxadiene and its isomer to TaxE9 (Figure 1B). Iso-taxadiene was detected as a cyclization byproduct of GGPP via GCMS, with mass spectra, retention time, and product ratio to taxadiene matching those previously reported,[18] purified via preparative-scale HPLC, and used in feeding experiments. As before, when feeding taxadiene, multiple products were observed. However, when feeding was performed with iso-taxadiene, only the T5OH product was observed. As epoxidation of iso-taxadiene cannot feasibly yield T5OH (Figure 1A), it can be reasoned that this isomer of taxadiene undergoes radical rebound hydroxylation.

Significantly, through examination of the reaction scheme for radical rebound, it can be seen that if the two isomers share the same common radical intermediate **2**, they should theoretically yield identical product profiles regardless of expression system. However, as taxadiene oxidation does not result in the same product distribution as iso-taxadiene (Figure 2B), we can deduce that these substrates undergo different reaction mechanisms, with distinct reaction intermediates. These assertions—of differing mechanisms and radical rebound for iso-taxadiene—when taken together, aid in establishing that taxadiene undergoes epoxidation by 5αCYP.

Evaluation of Alternative Oxidants. We next sought to further understand the catalytic cycle of 5αCYP and elucidate why the two isomers undergo different oxidation processes. P450s have been proposed to utilize multiple reactive species to perform oxidative reactions, with the two supported by the most evidence being the so-called Cpd0 (Fe^{3+}-OOH) and CpdI (*vide supra*;[15] Figure S2). Cpd0 has been reported to favor epoxidation, and its use would support an epoxidation-based mechanism. Additionally, it has been reported that when P450s utilize the same oxidizing specie (CpdI) to catalyze both hydroxylation and epoxidation, the reaction path is decided primarily by the ionization energy of the substrate rather than enzyme properties.[22−24] Such substrate-driven selectivity would further bolster the epoxidation-based mechanism as it aids in explaining the different paths the alternative taxadiene isomers take *en route* to final products.

A single highly conserved acid−alcohol pair has been demonstrated to be critical for the transition from Cpd0 to CpdI (Figure S2).[23] If turnover rates for different substrates decrease proportionally upon mutagenesis of the conserved alcohol (typically a threonine, here Thr310) to a nonpolar amino acid, it is likely they utilize the same oxidizing species. We thus mutagenized 5αCYP and compared the catalytic activity between a T310A mutant and its wild-type parent on the taxadiene and its isomer. We observed that turnover in crude-lysates was reduced to less than 5% of the wild type enzyme for both isomers, thus implicating a common oxidant and supporting the substrate control hypothesis. This result discounts the role of Cpd0 in oxidation by 5αCYP and adds further weight to the proposal that enzyme preference for epoxidation versus hydroxylation is primarily substrate-driven.

Further Functionalization via Acetylation Does not Alter Product Selectivity. It has been proposed that downstream functionalization may drive production or influence product distribution, as products may be in equilibrium *in vivo*. We thus aimed to establish that the observed products are not in equilibrium *in vivo* and are incapable of isomerization to T5OH by examining the effect further enzymatic functionalization had on the selectivity of 5αCYP. To achieve this, we made use of a previously characterized acetyltransferase.[25] We first confirmed the activity of the acetyltransferase *in vitro* using crude lysates of *E. coli* strain TaxE14 crude lysates (Figure 5D). Following incubation of the crude lysate with T5OH, a new compound with GCMS retention time and mass spectra (Figure S7A) matching that of monoacetylated compound was obtained, confirming T5OH could be acetylated in our system to generate Taxa-4(20),11-dien-5alpha-yl acetate (T5Ac; Figure 5D).

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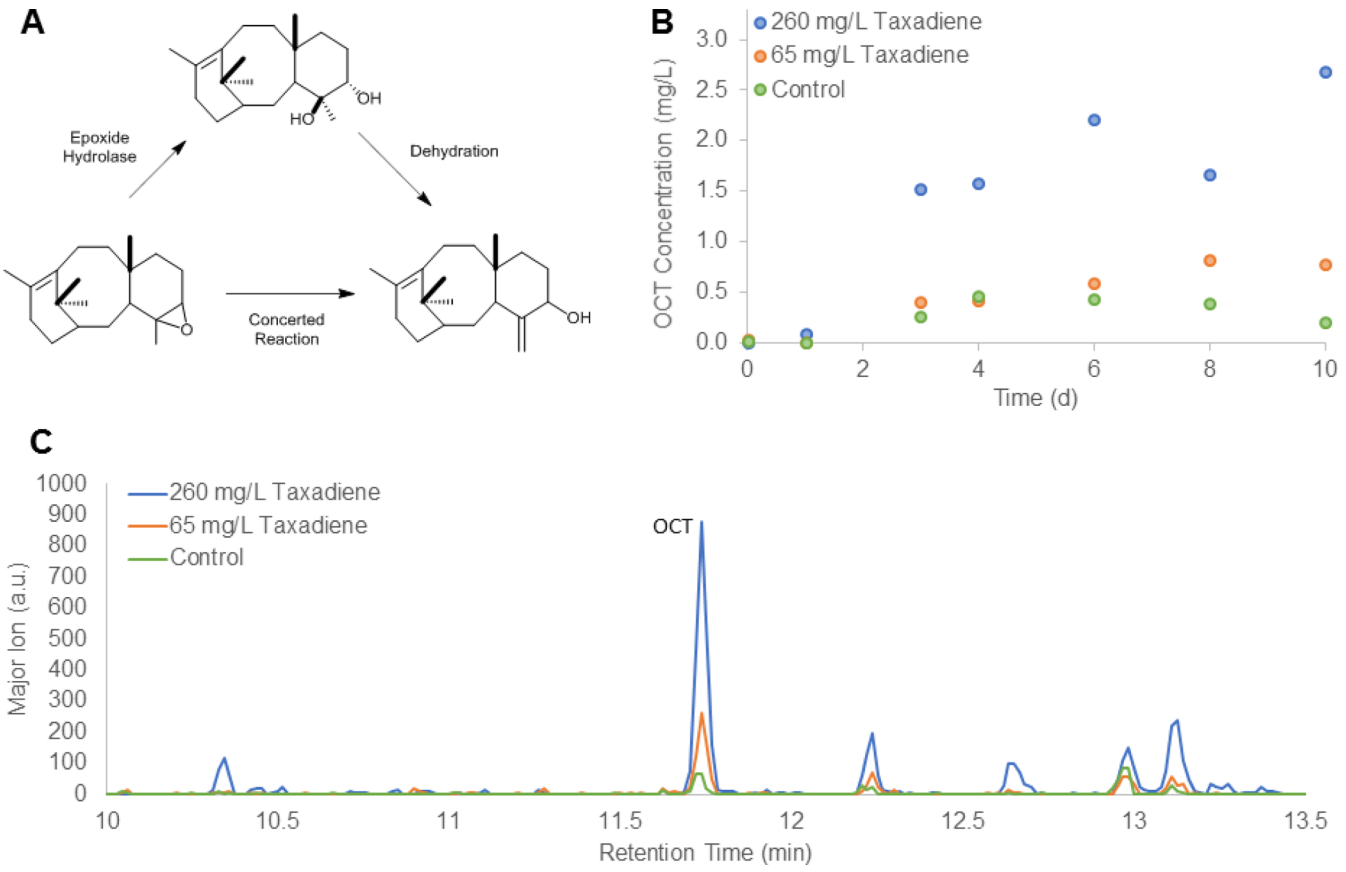

Figure 6. Formation of products in *Taxus cuspidata*. (A) Proposed routes from the putative epoxide intermediate **1** to the desired product, T5OH. Two routes are feasible. Upper route would utilize enzymes which have presecent within terpenoid biosynthesis including an epoxide hydrolase and a dehydratase, both of which have characterized enzymes known for other terpenoids (citations). (B) Time course study of OCT formation by *T. cuspidata* cultures supplemented with exogenous taxadiene, displaying the formation of OCT occurs in native plant cells and can be increased when exogenous taxadiene is fed. (C) GCMS chromatogram of taxanes displaying OCT, shown with primary ion (191 *m*/*z*), obtained from plant cell cultures supplemented with exogenous taxadiene, displaying OCT formation.

We then cloned this enzyme into an *E. coli* strain expressing the entire biosynthetic pathway to generate TaxE14. Fermentation of this strain led to near-complete conversion of taxadiene-5α-ol to its acetylated form, without a similar decrease in the production of other products (Figure 5C). Furthermore, no dramatic change was observed in the total T5OH and T5Ac selectively following expression of the acetyltransferase (Figure S7). This effectively discounted both the proposal that these compounds may be in equilibrium and that utilizing a rapid-acting downstream enzyme may correct selectivity.

Conversion of Taxadiene in Native Plant Cultures. We next aimed to determine if OCT and other observed compounds are products of heterologous expression or can also be produced in the native host, *Taxus cuspidata*. For screening, methyl jasmonate induced *T. cuspidata* cell cultures were supplemented with exogenous taxadiene in order to boost generation of 5αCYP products. These cells were then screened for the appearance of the primary oxidation byproduct, OCT. A clear peak matching the retention time and mass spectra of OCT was present in all cultures, with the concentration in fed cells increasing with increased supplementation (Figure 6). This result demonstrates that products other than T5OH are in fact produced natively. The low quantity of OCT (0.5 mg/L) in the native host implies a more favorable product distribution for T5OH by 5αCYP, or further metabolism of OCT either through degradation or further oxidation to final products. For example, OCT may act as a precursor to compounds containing a C-3(11) bridge, of which there are 18 currently known.[17,26] Additionally, as OCT is produced both natively and heterologously and the selectivity of taxadiene oxidation is substrate controlled (see above), it is difficult to reconcile that product profiles are influenced by a reductase partner; however, the possible influence of various redox partners on substrate and product affinities and the concomitant changes to the observed product distribution cannot yet be predicted or discounted. Furthermore, improved selectivity of the epoxide degradation step may be a function of intracellular conditions or the presence of additional enzymes. Feasibly, two enzymatic routes are possible that may generate T5OH from epoxide **1**. The first consists of a two-step reaction catalyzed by an enzyme similar to lemonine epoxide hydrolase,[27] which cleaves the epoxide to vicinal diols. A second enzyme would then perform a dehydration reaction to form T5OH; such activity has precedent in terpenoid biosynthesis.[28] The alternative route, consisting of a single enzymatic step, would cleave the epoxide directly to the desired product (Figure 6A). As such an enzyme would enable the improved biosynthesis of taxol precursors in heterologous systems, their discovery warrants further study.

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CONCLUSION

This study provides compelling evidence for the existence of an epoxide intermediate in the mechanism of 5αCYP which poses a significant, and previously unknown, obstacle to high-level taxol biosynthesis. Though epoxide instability precluded its direct isolation and characterization, we were able to utilize indirect evidence to establish its existence. These include the following: (1) By varying hosts, media conditions, and solvents, we demonstrated a change in the oxidized taxadiene pools. (2) Identical products and similar product ratios were generated through chemical epoxidation and enzymatically by 5αCYP. (3) Product structures and analysis of possible mechanistic pathways show a clear route from the proposed epoxide intermediate. (4) Different product profiles were obtained upon feeding of two taxadiene isomers to whole cell 5αCYP catalysts, indicating substrate control on the reaction outcome that is not reconcilable with the previous assertions of taxadiene oxidation by 5αCYP. Other lines of evidence showing a deeper understanding of enzyme mechanics including support for CpdI as the primary oxidant, further supporting the substrate-driven selectivity preference, inability of further functionalization to shift product profiles, and the detection of multiple products in the native plant host yield further insights into the mechanism of the enzyme. Although instability of the putative epoxide precluding characterization *via* NMR seems at odds with the above findings of intermediate extraction from whole cells by C18 resin, consideration of the different time-scales for extraction, which derive from diffusional time scales, as compared to time scales required for purification and NMR characterization reconciles these contrasting observations.

Furthermore, the array of data in support of an epoxidation-based mechanism is more compelling than that previously used to support one based on radical rebound. Three primary lines of evidence were previously used to support the radical-rebound mechanism: (1) No free epoxide was detected. (2) 5αCYP is capable of utilizing alternative taxadiene isomers. And, (3) no isomerization of taxadiene isomers was observed. We are able to address each of these points as we show the thermochemical instability of the epoxide precludes its easy detection as it rapidly decomposes. Alternative isomers are not required to undergo the same oxidation process, and no isomerization between taxadiene isomers is required. Furthermore, the previously proposed, radical-based, allylic rearrangement is without precedent in previous literature, whereas epoxidation of terpenoids by P450s is well documented.[21] Through a proposal of the epoxide intermediate, we were able to rectify previous, conflicting, reports on the selectivity of 5αCYP.

In addition to offering improved understanding of 5αCYP, these insights enable future work in improving the biosynthetic yield of taxol and its analogues. This can be seen as, by assuming a radical-based mechanism, only a single route to improvement is available (P450-mutagenesis); however, with a substrate-driven epoxidation route, one is able to intervene to improve selectivity at multiple steps within the pathway. These locations include prior to the enzyme, by supplying different substrates, as demonstrated by our feeding of alternative isomers, and the addition of an enzyme capable of metabolizing the epoxide to the desired product.

METHODS

Strain Construction. *E. coli* strains TaxE0, TaxE1, TaxE9, and TaxE12 and the *S. cerevisiae* strain TaxS1 were previously constructed by Kang Zhou (National University of Singapore) and Chin Giaw Lim (Manus Bio, Cambridge Massachusetts, USA) in our laboratory.[3] TaxE12 contains the 5aCYP and *T. cuspidata* CPR domain as a fusion construct, as in previous literature.[1] TaxE14 was generated by modification of the plasmid from TaxE12 (pSC101 origin of replication) to include an operon containing the acetyltransferase, into and cloning into TaxE1.[1] The nonfused P450 expression strain, Tax15, was generated by replacing the linker domain of Tax12 fusion construct with a ribosomal binding site and a membrane targeting domain as was performed for 5αCYP;[1] no difference in selectivity was observed between TaxE14 and TaxE15 (Supporting Information Figure S9). *Y. lipolytica* strain TaxY1 was constructed by integrating the expression cassette harboring 5αCYP and CPR under the control of a TEF intron promoter. Strains used in this study are listed in Table S9.

Small Scale Fermentations. For cultures of *S. cerevisiae* or *Y. lipolytica*, a single colony of the desired strain was inoculated into 1 mL of YNB (yeast nitrogen base with casamino acids and ammonium sulfate, Difco, supplemented with 20g/L glucose) or YPD (10g/L yeast extract, 20 g/L peptone, 20g/L glucose) and cultured overnight (30°/250 rpm). A total of 20 μL of the overnight culture was used to inoculate 1 mL of the desired culture media (YPD or YNB) in a 14 mL Pyrex glass tube for culturing. For feeding experiments, taxadiene was fed exogenously upon inoculation (0.1 mg in 10 μL DMSO). Cultures were shaken (22°/250 rpm) for 5 days prior to analysis of product distribution.

For *E. coli* cultures, a single colony of *E. coli* was inoculated into LB media (10g/L tryptone, 5 g/L yeast extract, 10g/L NaCl, pH = 7) and shaken overnight (37°,250 rpm). A total of 20 μL of overnight culture was inoculated into 1 mL of small-scale culture media (5 g/L yeast extract, 10 g/L tryptone, 15g/L glycerol, 10g/L NaCl, 100 mM HEPES, pH 7.6), unless otherwise stated, in a 14 mL pyrex glass tube. Cultures were induced upon inoculation with 0.1 mM IPTG and shaken (22°/250) for 5 days prior to analysis of product distribution.

For feeding of taxadiene and iso-taxadiene, these compounds were added as a solution in DMSO (1% final culture volume, final concentration of 100 mg/L) at the time of inoculation. In the extractive-fermentative regime, *E. coli* strain TaxE12 was grown in the presence of 100 μL of DINP, 100 μL of dodecane, or 50 mg of C18 chromatography resin. All the plasmids constructed in this study were validated by means of sequencing

Preparation of Taxadiene, iso-Taxadiene, Compound 4, and Compound 5. A 13-L Bioflow bioreactor (New Brunswick) was used for semipreparative scale production of desired compounds. Seed cultures, generated by inoculating a single colony into 100 mL of LB and shaking overnight (37 °C, 250 rpm), were inoculated into 10 L of fermentation media (5 g/L yeast extract, 20 g/L glucose, 13.3 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 0.0084 g/L EDTA, 0.0025 g/L $CoCl_2$, 0.015 g/L $MnCl_2$, 0.0015 g/L $CuCl_2$, 0.003 g/L H_3BO_3, 0.0025 g/L Na_2MoO_4, 0.008 g/L $Zn(CH_3COO)_2$), 0.06 g/L Fe(III) citrate, 0.0045 g/L thiamine, 1.3 g/L $MgSO_4$, pH 7.0). The temperature was maintained at 22 °C, and the pH was maintained at 7.0 *via* addition of NaOH. For taxadiene and iso-taxadiene production, cultures were performed under anaerobic conditions to reduce cell density, aiding in product recovery. For oxygenated taxanes such as compound **4**, oxygen was supplied as sterile-filtered air at 1 L/min with agitation adjusted to maintain DO at 10%. Induction with 0.1 mM IPTG was performed when OD reached 0.8. Taxanes were obtained from culture broth in a one-step extraction using a C18 chromatography resin and a 100% ethyl acetate for elution (100 mL). The effluent was then dried to completion, suspended in acetonitrile, and used for HPLC purification.

Preparation of **4** for NMR characterization was performed by epoxidizing biologically produced taxadiene (HPLC purified) and refluxing in acetonitrile for 4 h to increase the quantity of compound **4** present. Semipreparative HPLC purification was performed using a Supelco Discovery C18 (25 cm × 10 mm × 5 μm) HPLC column. Compound **4** was then purified under isocratic conditions (60% acetonitrile in water at 6 mL/min) with **4** possessing an elution time of 42 min. Taxadiene and iso-taxadiene were purified under isocratic

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conditions (90% acetonitrile in water, 6 mL/min) and had elution times of 19.1 and 20.2 min, respectively.

Chemical Reaction Experiments. In a typical mCBPA reaction,[29] taxadiene (1 mg, 0.0036 mmol) was weighed into a 14 mL pyrex glass tube containing CH_2Cl_2 (1 mL), $NaHCO_3$ (0.5 mg, 0.05 mmol). A magnetic stir bar was added and placed in a salt water/ice bath on a magnetic stir plate. The reaction was initiated with the addition of mCPBA (20 μL of a 25 mmolar stock, calculated assuming a nominal purity of 70%) and was allowed to proceed for 2 h. The crude product mixture was washed twice with saturated sodium carbonate and once with NaS_2O_3. The organic layer was then removed and dried to completion and the products subjected to analysis. Direct analysis of the organic layer via GCMS yielded identical product ratios, indicating wash steps did not alter product distribution.

A typical DMDO reaction was carried out by adding 0.34 mg of taxadiene (0.00125 mmol) to 250 μL of EA and 250 μL of water (buffered with 0.66 mg sodium carbonate) with 10 μL of acetate. Oxone in water (4.8 mg in 1 mL) and a solution of acetone and sodium carbonate in water (18 μL and 0.66 mg, respectively, in 1 mL) were added dropwise as separate solutions over 2 h. Sodium carbonate was chosen as the final buffering reagent as the reactions exhibited maximum yield when operated at high pH. Additionally, scaling up or transition to an acetone-free DMDO preparative method[30] did not alter the product distribution observed via GCMS or HPLC. HPLC analysis of chemical reaction experiments was performed using a Waters Spherisorb ODS2 (25 cm × 4.6 mm × 3 μm) column using a gradient from 50% to 70% acetonitrile in water.

For acid hydrolysis of compound **5**, 0.1 mg of HPLC-purified **5** was incubated in the presence of 5% HCl at 37 °C for 1 h. Following incubation, the products were analyzed via GCMS.

Crude Lysate Enzymatic Assays. Crude lysates for enzymatic assays were performed by diluting cells to an equal optical density followed by lysis with BPER II. NADPH cycling[31] was used to ensure adequate NADPH supply by supplying NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase when employing P450s. Reaction was initiated upon the addition of taxadiene, with reactions performed without additional supplied NADPH showing no turnover. Products were extracted using ethyl acetate and analyzed via GCMS.

OCT Detection in Plant Cell Cultures. *Taxus cuspidata* cell suspension cultures were grown in a B5 sucrose medium. Two days after passaging, cells were induced with 40 μL/L methyl jasmonate and supplemented with the appropriate quantity of taxadiene. Cells were harvested 7 days postinduction and extracted, and isoprenoids were quantified.

Quantification of Isoprenoids. At the conclusion of fermentations, 200 μL of fermentation broth was sampled and added to 200 μL of ethyl acetate containing an internal standard and 100 μL of 0.1 mm glass beads. This solution was then vortexed at 4 °C for 30 min and centrifuged at 18 000*g* for 10 min. The top, organic, layer was extracted and 1 μL was subjected to analysis via GCMS. The GCMS consisted of a Varian Saturn 3800 GC containing an HP-5 ms (Agilent Technologies, USA) column and a Varian 2000 MS, with helium at a flow rate of 1 mL/min as the carrier gas. The injector and transfer lines were maintained at 250 °C. For oxygenated taxanes, the column oven was maintained at 100 °C for 1 min and increased at a rate of 15 °C/min to 175 °C, then at 4 °C/min to 220 °C, then at 50 °C/min to 290 °C, and held at this temperature for 1 min. For analysis of taxadiene isomers, the column oven was maintained at 100 °C for 1 min and increased at a rate of 15 °C/min to 165 °C, then at 2 °C/min to 170 °C, held at this temperature for 7 min, increased at 50 °C/min to 290 °C, and held at this temperature for 1 min. Caryophyllene (90 mg/L) was used as an internal standard. Analysis of dodecane and DINP was performed by diluting 100-fold into an internal standard.

NMR Characterization of 4. All NMR spectra were collected using a 500 MHz Varian INOVA spectrometer. All spectra were detected using a z-gradient 5 mm inverse broadband probe. Full experimental procedures may be found in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00767.

Supporting Figures S1−S10; Tables S1−S3; full methods for NMR of compound **4**; and ^{1}H NMR, ^{13}C NMR, gCOSY, HSQC, and gHMBC of compound **4** (PDF)

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Author Contributions

S.E. and G.S. conceived the project. K.Z., K.Q., J.K., S.E., and G.S. designed the experiments, analyzed the results, and wrote the manuscript. K.Z., K.Q., J.K., and S.E. did all the experiments excluding the NMR. J.S. performed and analyzed the NMR.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ajikumar, P. K., Xiao, W.-H., Tyo, K. E. J., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B., and Stephanopoulos, G. (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. *Science 330*, 70−74.

(2) Nicolaou, K. C., Yang, Z., Liu, J. J., Ueno, H., Nantermet, P. G., Guy, R. K., Claiborne, C. F., Renaud, J., Couladouros, E. a, and Paulvannan, K. (1994) Total synthesis of taxol. *Nature 367*, 630−634.

(3) Zhou, K., Qiao, K., Edgar, S., and Stephanopoulos, G. (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat. Biotechnol. 33*, 377−383.

(4) Mendoza, A., Ishihara, Y., and Baran, P. S. (2012) Scalable enantioselective total synthesis of taxanes. *Nat. Chem. 4*, 21−25.

(5) Soliman, S., and Tang, Y. (2015) Natural and engineered production of taxadiene with taxadiene synthase. *Biotechnol. Bioeng. 112*, 229−235.

(6) Naill, M. C., and Roberts, S. C. (2005) Flow cytometric identification of paclitaxel-accumulating subpopulations. *Biotechnol. Prog. 21*, 978−983.

(7) Lenka, S. K., Nims, N. E., Vongpaseuth, K., Boshar, R. a., Roberts, S. C., and Walker, E. L. (2015) Jasmonate-responsive expression of paclitaxel biosynthesis genes in Taxus cuspidata cultured cells is negatively regulated by the bHLH transcription factors TcJAMYC1, TcJAMYC2, and TcJAMYC4. *Front. Plant Sci. 6*, 1−13.

(8) Croteau, R. (1996) A cDNA Clone for Taxadiene Synthase, the Diterpene Cyclase That Catalyzes the Committed Step of Taxol Biosynthesis. *J. Biol. Chem. 271*, 9201−9204.

(9) Hefner, J., Rubenstein, S., and Ketchum, R. (1996) Cytochrome P450-catalyzed hydroxylation of taxa-4 (5),11(12)-diene to taxa-4(20),11(12)-dien-5a-o1: the first oxygenation step in taxol biosynthesis. *Chem. Biol. 4*, 479.

(10) Jennewein, S., Long, R. M., Williams, R. M., and Croteau, R. (2004) Cytochrome P450 taxadiene 5??-hydroxylase, a mechanistically unusual monooxygenase catalyzing the first oxygenation step of taxol biosynthesis. *Chem. Biol. 11*, 379−387.

(11) Jennewein, S., Rithner, C. D., Williams, R. M., and Croteau, R. B. (2001) Taxol biosynthesis: taxane 13 alpha-hydroxylase is a

DOI: 10.1021/acschembio.5b00767
ACS Chem. Biol. XXXX, XXX, XXX−XXX

cytochrome P450-dependent monooxygenase. *Proc. Natl. Acad. Sci. U. S. A. 98*, 13595−600.
(12) Schoendorf, a, Rithner, C. D., Williams, R. M., and Croteau, R. B. (2001) Molecular cloning of a cytochrome P450 taxane 10 beta-hydroxylase cDNA from Taxus and functional expression in yeast. *Proc. Natl. Acad. Sci. U. S. A. 98*, 1501−6.
(13) Jennewein, S., Rithner, C. D., Williams, R. M., and Croteau, R. (2003) Taxoid metabolism: Taxoid 14β-hydroxylase is a cytochrome P450-dependent monooxygenase. *Arch. Biochem. Biophys. 413*, 262−270.
(14) Dejong, J. M., Liu, Y., Bollon, A. P., Long, R. M., Jennewein, S., Williams, D., and Croteau, R. B. (2006) Genetic engineering of taxol biosynthetic genes in Saccharomyces cerevisiae. *Biotechnol. Bioeng. 93*, 212−24.
(15) Modi, A. R., and Dawson, J. H. (2015) Oxidizing Intermediates in P450 Catalysis: A Case for Multiple Oxidants. *Adv. Exp. Med. Biol. P450*, 63−75.
(16) Rittle, J., and Green, M. T. (2010) Cytochrome {P}450 Compound {I}: Capture, Characterisation, and {C−H} Bond Activation Kinetics. *Science (Washington, DC, U. S.) 330*, 933−937.
(17) Rontein, D., Onillon, S., Herbette, G., Lesot, A., Werck-Reichhart, D., Sallaud, C., and Tissier, A. (2008) CYP725A4 from yew catalyzes complex structural rearrangement of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. *J. Biol. Chem. 283*, 6067−75.
(18) Guerra-Bubb, J., Croteau, R., and Williams, R. M. (2012) The Early Stages of Taxol Biosynthesis: An Interim Report on the Synthesis and Identification of Early Pathway Metabolites. *Nat. Prod. Rep. 29*, 683−696.
(19) Girhard, M., Machida, K., Itoh, M., Schmid, R. D., Arisawa, A., and Urlacher, V. B. (2009) Regioselective biooxidation of (+)-valencene by recombinant E. coli expressing CYP109B1 from Bacillus subtilis in a two-liquid-phase system. *Microb. Cell Fact. 8*, 36.
(20) Paddon, C. J., Westfall, P. J., Keasling, J. D., et al. (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature 496*, 528−532.
(21) Corey, E. J., Virgil, S. C., and Sarshar, S. (1991) New mechanistic and stereochemical insights on the biosynthesis of sterols from 2,3-oxidosqualene. *J. Am. Chem. Soc. 113*, 8171−8172.
(22) Kumar, D., Karamzadeh, B., Sastry, G. N., and De Visser, S. P. (2010) What factors influence the rate constant of substrate epoxidation by compound i of cytochrome P450 and analogous iron(IV)-oxo oxidants? *J. Am. Chem. Soc. 132*, 7656−7667.
(23) Vaz, A. D., McGinnity, D. F., and Coon, M. J. (1998) Epoxidation of olefins by cytochrome P450: evidence from site-specific mutagenesis for hydroperoxo-iron as an electrophilic oxidant. *Proc. Natl. Acad. Sci. U. S. A. 95*, 3555−3560.
(24) Jin, S., Makris, T. M., Bryson, T. a, Sligar, S. G., and Dawson, J. H. (2003) Epoxidation of Olefins by Hydroperoxo - Ferric Cytochrome P450. *J. Am. Chem. Soc. 125*, 3406−3407.
(25) Walker, K., Ketchum, R. E., Hezari, M., Gatfield, D., Goleniowski, M., Barthol, a, and Croteau, R. (1999) Partial purification and characterization of acetyl coenzyme A: taxa-4(20),11(12)-dien-5alpha-ol O-acetyl transferase that catalyzes the first acylation step of taxol biosynthesis. *Arch. Biochem. Biophys. 364*, 273−279.
(26) Itokawa, H. (2003) Taxoids occurring in the genus Taxus. *Taxus: the genus Taxus 4*, 15−16.
(27) Hopmann, K. H., Hallberg, B. M., and Himo, F. (2005) Catalytic mechanism of limonene epoxide hydrolase, a theoretical study. *J. Am. Chem. Soc. 127*, 14339−14347.
(28) Brodkorb, D., Gottschall, M., Marmulla, R., Lüddeke, F., and Harder, J. (2010) Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. *J. Biol. Chem. 285*, 30436−30442.
(29) Kim, C., Traylor, T. G., and Perrin, C. L. (1998) MCPBA epoxidation of alkenes: Reinvestigation of correlation between rate and ionization potential. *J. Am. Chem. Soc. 120*, 9513−9516.
(30) Dematteo, P. W., Rasha, a, and Enquist, J. a. (2013) Simplified Preparation of Dimethyldioxirane (DMDO). *Org. Synth. 90*, 350.
(31) Ghigo, D., Riganti, C., Gazzano, E., Costamagna, C., and Bosia, A. (2006) Cycling of NADPH by glucose 6-phosphate dehydrogenase optimizes the spectrophotometric assay of nitric oxide synthase activity in cell lysates. *Nitric Oxide 15*, 148−153.

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