MOLECULAR CLONING AND CHARACTERIZATION OF THREE ENZYMES INVOLVED IN TAXOL/TAXOID BIOSYNTHESIS: TAXOID 2α-HYDROXYLASE, TAXOID 7β-HYDROXYLASE, AND TAXOID 5α-O-ACETYLTRANSFERASE

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MYDOANH CHAU find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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The highly functionalized diterpenoid Taxol, derived from yew (Taxus) species, is a potent antineoplastic drug used successfully in the treatment of a wide variety of cancers. The limited natural source of this drug has prompted the development of alternative production methods, such as semisynthesis from more available taxoid precursors isolated from needles of various Taxus species, which serve as the current source of Taxol. Thus, for the foreseeable future, the supply of Taxol must continue to rely upon biological methods of production and improvements to this biosynthetic process in yew or derived cell cultures requires a full understanding of the Taxol/taxoid biosynthetic pathways.

The Taxol biosynthetic pathway consists of approximately 20 enzymatic steps from the primary intermediate geranylgeranyl diphosphate, which is cyclized to taxa-4(5),11(12)-diene. This parental olefin then undergoes a series of at least nine cytochrome P450-mediated oxygenations, five acylations, and several other modifications en route to Taxol. To probe for mid-pathway cytochrome P450 hydroxylases, a 'surrogate' substrate, taxusin (taxadien-5α,9α,10β,13α-tetraol tetraacetate), was employed to functionally screen a family of cytochrome P450 oxygenases originating from Taxus cell cDNA libraries. This screen led to the

identification of a taxoid 2α-hydroxylase and a taxoid 7β-hydroxylase, both of which are capable of converting taxusin to their respective derivatives, as well as the reciprocal conversion of their respective pentaol tetraacetate products to a common hexaol tetraacetate.

Additionally, a comparable functional screen using polyhydroxylated substrates was performed with similarly isolated Taxus acyltransferase clones, which led to the identification of another taxoid 5α-O-acetyltransferase. However, this enzyme demonstrated several differences from the previously-isolated 5α-O-acetyltransferase; both are capable of converting taxadien-5α-ol to taxadien-5α-yl acetate but display different regiospecificity with polyhydroxylated taxoid substrates. Examination of kinetic profiles suggests that the previously-isolated 5α-O-acetyltransferase prefers to acetylate taxadien-5α-ol, whereas this new acetyltransferase has a broader specificity and may be responsible for the abundance of acetylated taxoids.

The isolation of these enzymes and their corresponding genes is important in gaining an understanding of Taxol biosynthesis. Also, manipulation of these genes could allow increased production of Taxol, as well as the generation of other useful taxoid derivatives for subsequent studies.

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To my Robert,
thank you for loving me

CHAPTER ONE

GENERAL INTRODUCTION

Taxus (yew) species are known to biosynthesize a wide range of natural products called taxoids (taxane diterpenoids), of which more than 350 have now been identified [1]. The reason(s) for the biosynthesis of such a variety of natural products is not well understood. However, several of these taxoids have been shown to have roles in defense, such as antifeedant [2] and antifungal [3, 4] activities, and some taxoids, called taxines, are poisonous to mammals [5, 6]. Additionally, several taxoids have also been shown to possess medicinal properties such as anti-inflammatory [7] and anticancer [8, 9] activities. The most well-studied taxoid, Taxol (paclitaxel, Fig. 1), has been demonstrated to be an effective anticancer agent against a number of different cancers, including breast, ovarian, prostate, non-small cell lung, head, and neck cancers, and AIDS-related Kaposi's sarcoma [10-13]. Currently, combination therapies with other anticancer agents, such as gemcitabine [14] and platinum-containing drugs [15], are showing improved efficacy against these and other cancers, such as gastric cancer [16].

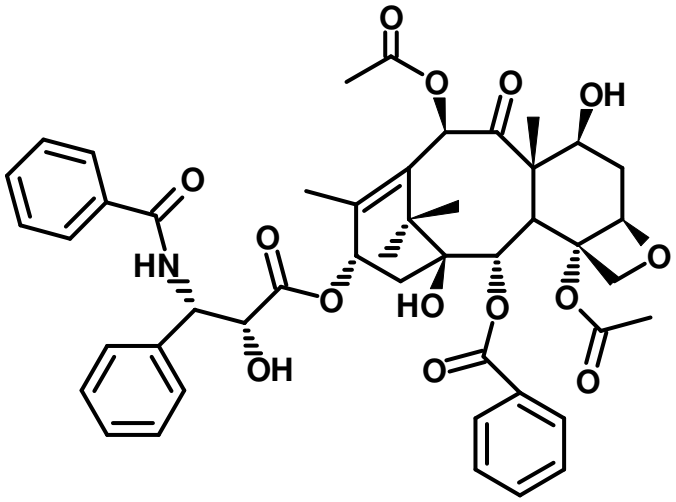

Figure 1: Taxol (paclitaxel), a potent anticancer drug first isolated from the Pacific yew.

Additionally, a significant amount of research is being conducted to improve the efficacy of Taxol. One factor which has limited the utility of Taxol is its low solubility in water, which has complicated the intravenous infusion process used in the treatment of cancer patients. The current vehicle for drug administration is Cremophor EL (polyoxyethylated castor oil) and ethanol, which is diluted with a suitable parenteral fluid. The side effects associated with this infusion treatment include reduction in white and red blood cell counts and infection, as well as, hair loss, nausea and vomiting, joint and muscle pain, nerve pain, numbness in the extremities and diarrhea [17]. As an attempt to increases the solubility of Taxol, reduce the side effects associated with administration, and increase the potency against neoplastic cells, several research groups have synthesized second generation Taxol derivatives. Many of these new compounds have demonstrated increased efficacy against the above mentioned cancers [18-20]. These second generation drugs are designed to increase the solubility and improve binding to tubulin as the principal mode of action in arresting cell growth [21].

History of Taxol

In the 1960's, the National Cancer Institute funded a joint program with the USDA to screen plant extracts for anticancer activity. The search included the Pacific Northwest where numerous plant samples were collected, extracted, and screened against various cancer cell lines. From this screen, extracts from the Pacific yew bark showed antitumor activity. The principal compound was not identified until many years later (in 1971 by M. Wani and M. Wall) as Taxol, now commonly known as paclitaxel (Fig. 1). Taxol is a complex diterpene with a rigid tricyclopentadecane $[9.3.1.0]^{3,8}$ skeleton, that bears two free hydroxyl groups, a carbonyl, two acetate groups, a benzoyl group, a complex N-containing sidechain, and an unique oxetane ring

[9]. The difficulties associated with the extraction and purification of Taxol, mainly associated with the low yields from yew bark, nearly halted the development of this drug. It was not until 1979 that Taxol was advanced as a candidate for development by NCI, when its unique mode of action was identified by S. Horwitz [21]; Taxol was found to stabilize microtubules by promoting assembly and preventing disassembly. This mechanism was different than the classical anticancer drugs, like the vinca alkaloids (vincristine and vinblastine) and colchicines, which inhibit the polymerization of microtubules. Scientists immediately recognized this unique mode of cytotoxicity as a new means of treating cancer.

The mechanism of action of Taxol involves binding to a pocket in the β-subunit of tubulin, thus promoting polymerization and inhibiting depolymerization. The crystal structure of the Taxol-bound tubulin dimer, using electron crystallography, showed Taxol to be positioned between the α-helices H1, H6, and H7, the H6-H7 loop, β-strands B8-B10, and the B7-B9 M-loop [22, 23]. In addition to the rigid taxane core, the pharmacophores responsible for tubulin binding are the unusual oxetane ring, the C2-benzoate, the C4-acetate, and the C13-N-benzoylphenylisoserine side chain. The reason Taxol inhibits microtubule depolymerization is not yet known; however, it has been proposed that Taxol interferes with the lateral contacts between protofilaments, since it interacts at the N-terminal end of the M-loop which is involved in lateral interactions in microtubules [24]. Another model suggests that Taxol-induced stabilization is due to the strengthening the lateral bonds by effecting the M-loop during the loss of the γ-phosphate, thus counteracting effects of GTP hydrolysis [25].

In addition to microtubule stabilization, a number of other Taxol-associated cytotoxic mechanisms have been described. Taxol can induce the hyperphosphorylation of Bcl-2 (an anti-apoptotic protein) to promote apoptosis [26]. The activation of cell cycle controlling enzymes,

such as cdc2 (cell division control-2) kinase [27] and the induction of TNF-α, a cytokine shown to be cytotoxic towards cancer cells [28], all contribute to apoptosis. Taxol also inhibits arylamine N-acetyltransferase activity (NAT) and gene expression; increased NAT activity has been observed in some types of chemical carcinogenesis and Taxol acts as an uncompetitive inhibitor of this enzyme [29].

Taxol Supply Problem

Since Taxol has gained wide use as an effective anticancer drug, the supply of this natural compound has been an issue. Because of the low levels of Taxol in Taxus brevifolia (Nutt.) bark, numerous trees were required to produce a sufficient supply; the destruction of about eight trees is necessary to provide sufficient Taxol to treat a single patient [30]. The environmental impact of destructive bark harvesting on this slow growing natural resource promoted concern from the medical community regarding the long-term availability of this drug. As a result, alternative methods of producing the drug were sought, such as total synthesis (beginning with a simple precursor), semisynthesis (beginning with a highly functionalized, more readily available, intermediate), and culturing of certain endophytic fungi and Taxus cells to produce high levels of Taxol.

Total syntheses

Nicolau and Holton were the first to independently publish the total syntheses of Taxol from relatively simple, commercially available starting materials. Holton used a linear strategy, starting with the AB rings, followed by the addition of the C- and D-rings. This synthesis originates from (+)-β-patchoulene oxide in order to control stereochemistry of the carbon

skeleton en route to Taxol. This synthesis was achieved in 42 steps with a final yield of 0.6% [31, 32]. Nicolau's synthesis of Taxol involved a convergent strategy for generation of the functionalized A- and C-rings; this synthesis was achieved in 35 steps with a final yield of 0.06% [33, 34]. Additionally, Nicolau's approach generated a racemic mixture which required an additional final purification step.

Since these initial achievements, a number of other groups have devised total syntheses of Taxol. Danishefsky's approach was different from that of previous researchers, in that the oxetane ring was formed early and was maintained through the synthesis beginning with moderately functionalized A- and C-ring precursors. This synthesis was also performed in a convergent manner consisting of 24 steps with a final yield of 0.02% [35, 36]. Other researchers have also achieved the total synthesis of Taxol including the groups of Wender [37, 38], Kuwajima [39, 40], and Mukaiyama [41]. Although total syntheses of Taxol are great synthetic achievements, the low yields of these lengthy reaction sequences preclude commercial viability.

Semisynthesis

One of the major breakthroughs in addressing the Taxol supply problem was the isolation of the relatively abundant, highly functionalized taxoid metabolites, 10-deacetylbaccatin III and baccatin III, from the needles (a renewable source) of the English yew (T. baccata). These potential intermediates possess most of the required functional groups on the taxane core, except for the C13-N-benzoylphenylisoserine side chain. This finding prompted scientists to devise semisynthetic strategies to synthesize Taxol. For example, Holton developed a method [42] in which 7-TES-protected baccatin III is coupled with the β-lactam side chain designed by Ojima

[43]. Although other research groups have devised related semisynthetic approaches, the Holton method is currently used commercially as the major route for the production of Taxol (Fig. 2).

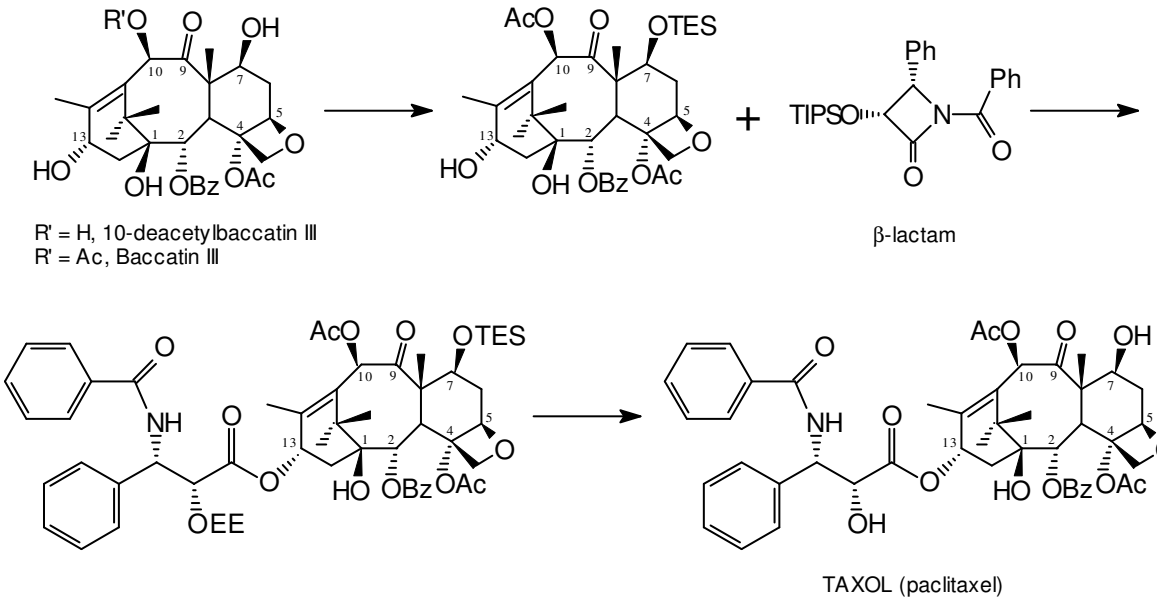

Figure 2: Outline of the Holton semisynthesis of Taxol (paclitaxel) from the natural product 10-deacetylbaccatin III and the synthetic β-lactam precursor of the N-benzoyl phenylisoserine side chain. TIPS, triisopropylsilyl; TES, triethylsilyl; EE, ethoxyethyl.

Endophytic fungi

Taxomyces andreanae is an endophytic fungus associated with the Pacific yew (T. brevifolia, and it was originally isolated from phloem (inner bark) tissue. This fungus was grown in semi-synthetic liquid media and was reported to produce Taxol. Radiolabeled precursors of Taxol, [1-^{14}C]acetic acid and L-[U-^{14}C]phenylalanine, were fed to these cultures and [^{14}C]Taxol was identified as a product [44]. Subsequently, a number of other groups identified other types of endophytic fungi associated with the Taxus species which are also reportedly capable of producing Taxol [45, 46]. These findings suggest fungal production as an

alternative source of Taxol; however, the low production yields have limited commercial development of this potential source.

Taxus cell culture

Taxus cell cultures produce Taxol and related taxoids in modest yield, and at least one commercial process for Taxol production, through large-scale culturing of Taxus suspension cell cultures, has been developed. These cultures originate from either embryo or needle explants which are cultured on callus induction media and eventually transferred to suspension culture media. When the cultures are elicited with methyl jasmonate, they produce up to 23 mg/L of Taxol [47]. Taxol and other taxoids are excreted into the medium, simplifying isolation, and the suspension cells are then subcultured in fresh media.

Although the semisynthetic approach using yew needle extracts has largely addressed the supply issue and eliminated destructive harvest of yew trees, the process has shortcomings. Preparation of advanced taxoid metabolites, such as baccatin III and 10-deacetylbaccatin III, from yew needle extracts requires purification from other plant metabolites, and the side chain must still be added by expensive synthetic means. A better approach would be to generate Taxol directly in high yields in Taxus cell cultures due to ease of isolation, and in theory, a more controllable system. Taxus cells express Taxol biosynthetic genes, and an understanding of the pathway, enzymology, and genetic basis for Taxol formation should permit the targeting of the rate-controlling steps for genetic engineering to achieve higher Taxol production levels. For the foreseeable future, Taxol supply will continue to rely on biological systems of production and an understanding of the biochemistry and molecular genetics of Taxol formation will be essential for improving yields and could be most useful in the production of second generation drugs.

Taxol Biosynthesis

The Taxol biosynthetic pathway (Fig. 3) is predicted to consist of twenty steps from the primary isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate. To date, more than half of the enzymes and genes involved in this pathway have been defined. This effort entails demonstrating the target activity in Taxus extracts, identifying substrates and products, where possible by feeding studies, and then isolating the corresponding genes. Taxus cell cultures elicited with methyl jasmonate for Taxol production were used as the source of biosynthetic enzymes and for generating gene libraries, including a differential display library [48] and an EST library [49]. In addition, some genes have been isolated through classical homology-based approaches [50]. Two groups of gene families have been isolated encoding, acyltransferases and cytochrome P450 oxygenases, each family consisting of about twenty unique clones. For functional screening of enzymes relevant to the Taxol pathway, the acyltransferases were expressed in Escherichia coli and the cytochrome P450 oxygenases were expressed in Saccharomyces cerevisiae or the Spodoptera-baculovirus system.

The Taxol pathway (Fig. 3) begins with the formation of the universal precursor of diterpenoids, geranylgeranyl diphosphate (GGPP), by GGPP synthase from the C_5 isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate [51]. The first committed step in the Taxol pathway is the cyclization of GGPP to taxa-4(5),11(12)-diene by the terpene cyclase, taxadiene synthase, for which the native enzyme and corresponding gene have been described [52, 53].

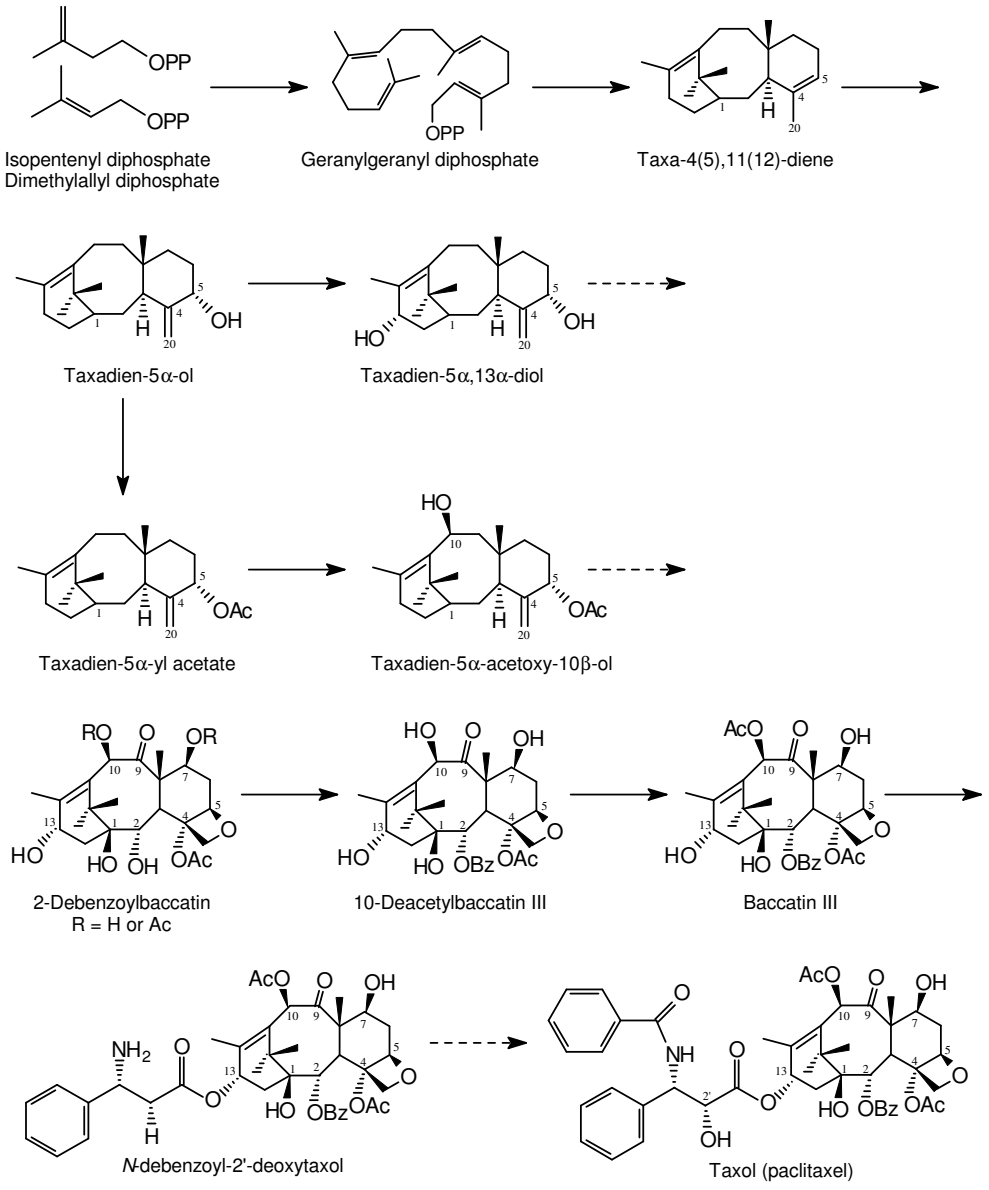

Figure 3: Scheme of the elucidated steps of the Taxol biosynthetic pathway. Shown is the cyclization of geranylgeranyl diphoshate to taxa-4(5),11(12)-diene and the subsequent hydroxylations and acylations en route to Taxol. Broken arrows indicated multiple steps, some of which have not been elucidated.

Although many of the other enzymes of this pathway have been identified, the exact order of the steps catalyzed and the relevant intermediates are in many cases not precisely

known. There are at least nine cytochrome P450 mediated oxygenations and five acyl-CoA dependent acylations en route to Taxol. The proposed order of the acylations has been established based on the reactions catalyzed; however, their exact placement in the pathway sequence is still uncertain. The order of oxygenation steps has yet to be fully elucidated, although predictions have been made based on a survey of the oxygenation patterns of the naturally occurring taxoids [54] and on biochemical studies on the early pathway steps [55]. The proposed oxygenation order begins with C5 and C10, then C2, C9, and C13 (exact order uncertain), followed with C7, and finally C1. Based on these assumptions, taxa-4(5),11(12)-diene was used as a substrate with Taxus microsomes [55] to demonstrate the hydroxylation at C5 and to screen the family of recombinant cytochrome P450 enzymes which led to the identification of a taxadien 5α-hydroxylase clone [50]. Taxadien-5α-ol has also been demonstrated in cell-free enzyme systems [55, 56] to be either hydroxylated by a cytochrome P450 taxoid 13α-hydroxylase [57] to form taxadien-5α,13α-diol, or acetylated by a taxadien-5α-ol-O-acetyltransferase [58] to form taxadien-5α-yl acetate. C5-acetylation appears to be the earliest acylation on the Taxol pathway, and this acyl group is predicted to play in a role in the formation of the oxetane ring through acyl migration and ring expansion of the corresponding C4,C20-epoxide (Fig. 4). Additionally, taxadien-5α-yl acetate was also demonstrated in microsomal systems [55] to be hydroxylated to taxadien-5α-acetoxy-10β-ol, and the cDNA encoding this cytochrome P450 taxoid 10β-hydroxylase was subsequently isolated [48]. Both the taxoid 10β-hydroxylase and the 13α-hydroxylase clones were acquired by differential display of mRNA-reverse transcription-PCR (DD-RT-PCR) using Taxus cells induced for Taxol production as the transcript source.

Figure 4: Proposed biosynthetic scheme of the oxetane ring formation in late-stage taxoids. The 4(20)-ene-5α-ol functional groups is converted to the corresponding 4(20)-ene-5α-yl acetate, followed by epoxidation to the 4(20)-β-epoxy-5α-acetate, and then acetate migration and epoxide ring expansion to form the oxetane ring.

Four acylations appear to occur late in the taxol biosynthetic pathway after the eight taxane ring oxygenations have taken place. The taxane 2α-O-benzoyltransferase was identified using, 2-debenzoyl-7β,13α-diacetylbaccatin III, as a surrogate substrate to screen the family of Taxus acyltransferase clones. In a similar manner, the 10β-O-acetyltransferase cDNA clone was identified by the conversion of the abundant metabolite 10-deacetylbaccatin III to baccatin III; this activity having also been demonstrated in a cell free enzyme system from Taxus cells [59]. One of the more interesting structural features of Taxol is the C13-N-benzoylphenylisoserine side chain. Previous Taxus feeding studies had suggested that the side chain was constructed in two steps, the attachment of phenylisoserine to baccatin III followed by N-benzoylation (i.e., not as an intact N-benzoylphenylisoserine moiety) [60]. However, these suggestions are inconsistent with more recent studies with native cell free enzyme systems and recombinant enzyme activities observed from the taxoid acyltransferase clones. Thus, the side chain acyltransferase (clone denoted TAX7) preferentially couples β-phenylalanoyl-CoA to the C13-hydroxyl group of baccatin III to form N-debenzoyl-2′-deoxytaxol (phenylisoserinoyl-CoA is a less efficient substrate) [61]. The final acylation step involves 3′-N-benzoyltransfer to the side chain, and the enzyme and corresponding gene (clone denoted TAX10) have been described [62]. Based on cell free enzyme studies and the specificity of the recombinant enzymes, the biosynthesis of the

side chain appears to occur in three sequential steps, the attachment of β-phenylalanine to baccatin III, followed by 2′-hydroxylation and then N-acylation. Another interesting enzyme involved in C13-side chain assembly is the aminomutase, which converts α-phenylalanine to β-phenylalanine for ligation to the CoA ester and acyl transfer [63].

Although all of the acyltransferases and several of the P450 oxygenases of Taxol biosynthesis have been defined, there are at least six cytochrome P450-mediated reactions which have yet to be elucidated. These include the oxygenations C1, C2, C7, C9, and C2′, and the C4,C20-epoxidation. Additionally, the oxidation of the C9-hydroxyl to the carbonyl has not yet been biochemically described but precedent in the biosynthesis of other natural products suggests that this step may also be cytochrome P450-mediated (via the ketone hydrate). Also, an interesting reaction that has yet to be elucidated is the proposed C4,C20-epoxide ring expansion/C5-acetate migration thought to be involved in oxetane ring formation. Furthermore, the CoA ligases for activation of the respective acyl groups have yet to be described.

To search for the C1, C2, and C7 hydroxylases and the proposed C4,C20-epoxidase, by in vitro studies and screening of recombinant enzymes, suitable substrates were necessary. Based on the predicted order of oxygenation, the C2 and C7 hydroxylation reactions are proposed to occur in the middle of the Taxol biosynthetic pathway, so a moderately functionalized substrate was an appropriate choice. However, relevant taxoid intermediates of this type are not readily available, thus requiring the use of an accessible surrogate. A naturally occurring metabolite of Taxus heartwood, (+)-taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate) was therefore chosen for this purpose as a surrogate substrate to probe for these cytochrome P450 enzymes. This substrate was first tested with Taxus microsomes to demonstrate feasibility of the target conversions, and then used as a guide to screen the family of

Taxus cytochrome P450 clones for the corresponding activities. From these screens, two clones denoted 3156 and F31 were identified to hydroxylate taxusin to the respective, 2α-hydroxy and 7β-hydroxy derivatives. These clones and characterization of the corresponding recombinant enzymes are described herein.

Examination of a compendium of naturally occurring taxoids [1] reveals a large assortment of positionally-different acylation patterns and variety of acyl and aroyl substituents. These latter functional groups include acetyl, propionoyl and butyroyl esters, and their hydroxylated and methylated derivatives, tigloyl and benzoyl groups, amino acid derivatives, such as cinnamoyl, aminophenylpropanoyl (Winterstein's acid), and phenylisoserinoyl esters, as well as glycosyl groups; the vast majority of advanced taxoids are acetylated. To gain information about the origin of these various acetylated taxoids, the remaining uncharacterized recombinant Taxus acyltransferases were screened with a range of polyhydroxylated taxoid substrates to access positional specificity in the transfer reaction. This study attempted to identify new clones that were capable of acetylating the test substrates and to examine regioselectivity and kinetic properties in order to determine the possible roles of these enzymes in taxoid biosynthesis. This screen yielded two new clones that were capable of acetylating several taxoid substrates. Additionally, several previously characterized taxoid acyltransferases were shown to possess alternative, new activities. One acetyltransferase (denoted clone TAX19) had apparently comparable activity relative to the previously-described taxadienol-5α-O-acetyltransferase (clone TAX1) [58], and was chosen for more detailed study reported here.

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CHAPTER TWO

Taxol Biosynthesis: Molecular Cloning and Characterization of a Cytochrome P450 Taxoid 7β-Hydroxylase

Summary

Biosynthesis of the anticancer drug Taxol in yew species involves eight cytochrome P450-mediated oxygenations and four coenzyme A-dependent acylations of the diterpenoid core. A family of cytochrome P450 genes, obtained from a yew cell cDNA library, were functionally expressed and screened with taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate) as a surrogate substrate. One clone converted this substrate to an oxygenated derivative that was identified as 7β-hydroxytaxusin. The structure and properties of this 7β-hydroxylase are similar to those of other taxoid hydroxylases. Kinetic and binding assays indicated selectivity of the 7β-hydroxylase for polyoxygenated and acylated taxoid substrates, an observation consistent with the operation of this enzyme in the central portion of the Taxol biosynthetic pathway. Although the 7β-hydroxyl of Taxol is not essential for antimitotic activity, this functional group provides a convenient means for preparing taxoid derivatives.

Introduction

The highly functionalized diterpenoid Taxol (generic name paclitaxel, Figure 1), derived from yew (Taxus) species [1], is a potent mitotic inhibitor used successfully in the treatment of a wide variety of cancers [2, 3]. The limited supply of this drug from its natural source has prompted the development of alternative means of production [4]. Total syntheses of Taxol have been achieved by several elegant routes (see [5] for recent review) but the yields are too low to be practical, and the principal source of the drug is via semisynthesis [6] from advanced taxane diterpenoid (taxoid) precursors (e.g., 10-deacetyl baccatin III, Figure 1) isolated from needles of various Taxus species. Thus, for the foreseeable future, the supply of Taxol and its semisynthetically useful precursors [5] must continue to rely upon biological methods of production [4, 7]. Improvement of the biosynthetic process in intact yew or derived cell cultures [8, 9] should be based upon a full understanding of the pathway for Taxol formation, the enzymes which catalyze this extended sequence of reactions and their mechanisms of action, and the structural genes encoding these enzymes, especially those responsible for slow steps of the pathway.

The Taxol biosynthetic pathway consists of approximately 20 enzymatic steps from the primary intermediate geranylgeranyl diphosphate [10] which is cyclized, in the first committed step of the pathway, to taxa-4(5),11(12)-diene [11] to establish the core skeleton [12] (Figure 1). This parental taxane then undergoes a series of eight cytochrome P450-mediated oxygenations and several acylations, including C13 side-chain assembly and attachment, en route to Taxol [13, 14]. The predicted order of hydroxylation, based on the relative abundances of the several hundred defined taxoids bearing oxygen functions at the various positions, begins with C5 and

C10, then C2, C9 and C13, and later C7 and C1 [15]. Biochemical studies are consistent with the proposed order in the early part of the pathway, C5 and C10 hydroxylations, and C13 hydroxylation has been demonstrated [16, 17]. All of the core acylations have also been described, as have those involved in C13 side-chain assembly and transfer [18]; the latter appears to occur after construction of the oxetane ring (see Figure 1). The intermediate steps of the Taxol biosynthetic pathway remain largely undefined.

Because natural product biosynthetic genes of plant origin are rarely clustered [19], a range of reverse genetic and homology-based cloning strategies, as well as EST mining [13, 14], have been employed to isolate cDNAs encoding geranylgeranyl diphosphate synthase [10], taxadiene synthase [20] and all of the CoA-dependent acyl and aroyl transferases of the Taxol pathway [18, 21-24]. In the case of the cytochrome P450 oxygenases, an additional approach was taken based on differential display of mRNA-reverse transcription-PCR, using cultured Taxus cells induced for Taxol production as the transcript source [25], and with sorting of clones by sequence relatedness and functional expression [26]. These combined strategies yielded a family of about two dozen related oxygenases from which cDNAs encoding the cytochrome P450 taxoid 5α-hydroxylase [27], 10β-hydroxylase [26], 13α-hydroxylase [28], and a side-route taxoid 14β-hydroxylase [29] were obtained to confirm the early part of the Taxol biosynthetic pathway.

Intermediate oxygenation steps of the pathway (i.e., from the level of a taxadien triol onward) have been much more difficult to approach. This mid-section of the pathway is not defined in reaction order and the intermediates are not known or, if predicted, are not readily available for testing. This serious difficulty necessitates the preparation and evaluation of 'surrogate' substrates to explore these 'central' hydroxylations. In the present instance, taxusin

(the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol) was employed as a surrogate substrate to functionally evaluate the extant cytochrome P450 clones for taxoid C1, C2 and C7 hydroxylase activities. Taxusin is a prominent metabolite of yew heartwood [30, 31] in which it is considered a dead-end metabolite, not an intermediate in Taxol formation [15]; nevertheless, the natural occurrence in Taxus of a broad range of taxusin-like metabolites bearing additional oxygen functional groups at C1, C2 or C7 [31-33] encouraged the use of this material as an alternate substrate for the present purpose. This approach led to the acquisition and characterization of a seemingly regioselective taxoid-7β-hydroxylase presumed to operate in the middle section of the multi-step Taxol biosynthetic pathway.

Results and Discussion

Preliminary Studies

To determine whether taxusin might be a suitable surrogate substrate for identifying oxygenases relevant to taxoid metabolism, microsomes prepared from cultured Taxus cells were incubated with [^{3}H-acetyl]taxusin under standardized cytochrome P450 assay conditions [16, 17]. The resulting reaction products were extracted and separated by radio-HPLC to reveal several metabolites more polar than taxusin (i.e., presumptive hydroxylated derivatives), thereby suggesting that Taxus microsomes do contain cytochrome P450 enzyme(s) capable of oxygenating taxusin.

Functional Screening of Cytochrome P450 Clones

To test the ability of the encoded enzymes of the previously acquired family of cytochrome P450 clones [26] to oxygenate taxusin, we exploited the simplicity and reliability of heterologous expression of these cDNAs in Saccharomyces cerevisiae strain WAT11 which harbors a galactose-inducible NADPH-cytochrome P450 reductase from Arabidopsis thaliana that is required for efficient reductive coupling to the cytochrome [34]. This system also permits test of catalytic activity by in vivo feeding of taxoid substrates to the transformed yeast [26-29], thereby eliminating the need for microsome isolation in the preliminary functional screen.

Transformed yeast cells, confirmed to express the recombinant cytochrome P450s by CO-difference spectra [35] as previously described [26], were tested for the ability to oxygenate taxusin by overnight incubation with [^{3}H-acetyl]taxusin. Extracts of the overnight reaction mixture of the 19 clones evaluated were then analyzed by radio-HPLC, and it was shown that one clone (designated F31) was capable of the nearly quantitative conversion of taxusin to more polar products [i.e., one large radio peak flanked by two smaller radio peaks (data not shown)]. LC-MS analysis of this material revealed a mass spectrum of the principal product that was consistent with that of a hydroxytaxusin. Although the parent ion (P^+) at m/z 521 [taxusin MW of 504 plus O (mass 16) + H^+] was not observed, diagnostic fragment ions were observed at m/z 461 (P^+-CH_3COOH), 401 (P^+-$2CH_3COOH$), 359 (P^+-$2CH_3COOH$-CH_3CO), 341 (P^+-$3CH_3COOH$), 299 (P^+-$3CH_3COOH$-CH_3CO), and 281 (P^+-$4CH_3COOH$). MS analysis of the two minor products yielded parent ions and diagnostic fragment ions suggestive of hydrolysis products of both the taxusin substrate and the putative hydroxytaxusin product, presumably arising via hydrolytic metabolism in the yeast host. For this reason, the products generated from taxusin by cytochrome P450 clone F31, as well as those generated from taxusin by Taxus

microsomes (see above), were peracetylated prior to reanalysis by LC-MS and GC-MS. These analyses of the derivatized material revealed that the minor hydrolytic products had disappeared (by collapsing into either tetraacetylated substrate or pentaacetylated product), and that the major product of taxusin metabolism by clone F31 and by Taxus microsomes had the identical retention time by HPLC (33.6 min) and by GC (17.66 min) and the identical mass spectrum by APCI and electron impact ionization (Figure 2). By APCI MS, the presumptive acetylated hydroxytaxusin product did not yield a detectable parent ion at m/z 563 [acetoxytaxusin (MW = 562) + H^+]; however, diagnostic fragment ions were observed at m/z 503 (P^+-CH_3COOH), 443 (P^+-$2CH_3COOH$), 401 (P^+-$2CH_3COOH$-CH_3CO), 383 (P^+-$3CH_3COOH$), 341 (P^+-$3CH_3COOH$-CH_3CO), 323 (P^+-$4CH_3COOH$), 299 (P^+-$3CH_3COOH$-$2CH_3CO$), 281 (P^+-$4CH_3COOH$-CH_3CO), and 263 (P^+-$5CH_3COOH$). These data indicated that taxusin was converted to the same hydroxylated product by yeast harboring clone F31 and by the cytochrome P450(s) of Taxus cell microsomes.

Product Identification

To confirm the identity of the product generated from taxusin by cytochrome P450 clone F31, and to determine the position and stereochemistry of the newly appended hydroxyl group, the transformed yeast incubations were scaled up 25-fold, and the resulting product was isolated and chromatographically purified to yield about 1 mg of material (>95% purity by HPLC, free of hydrolysis products and underivatized) for NMR analysis (Table 1). The previously determined ^{13}C-NMR shift assignments for taxusin [36] aided in assigning the chemical shifts for the putative hydroxytaxusin. The ^{1}H-NMR spectrum of the putative hydroxytaxusin was also quite similar to that of taxusin, as expected; however, a unique doublet of doublets in the sample was

observed at 4.34 ppm, consistent with the chemical shift of a proton attached to a carbon bearing a hydroxyl group. The only protons that could exhibit this type of coupling are either at C2 or C7. 2D-NOESY and 2D-TOCSY experiments revealed the regiochemistry of the hydroxyl substituent and the complete ^{1}H assignments. The NOESY experiment showed the doublet of doublets (δ 4.34) to have correlations between H6α and H6β. These 2D-NMR data confirm the hydroxyl to be on the C-ring at C7. The stereochemistry of the C7 proton was determined by nuclear Overhauser effect difference (NOE-DIF) spectroscopy, which revealed strong correlations with the α-oriented protons H3 (δ 2.83) and H10 (δ 6.23), and those of the C9α-acetate methyl (δ 2.07), and moderate coupling with H6α (δ 2.09). The strong correlations with H3 and H10 (both α-face) would be exhibited only if H7 was α-oriented, since a β-oriented hydrogen could not be so coupled. The strong correlation with H6α also indicated H7 to be of the gauche conformation; consistent with α-stereochemistry (models show H6β and H7α to be nearly orthogonal). These correlations confirm that H7α is attached to a carbon bearing a β-hydroxyl group and, thus, that the product of clone F31 is 7β-hydroxytaxusin.

The proton spectrum also revealed the presence of what appeared to be a significant contaminant in the sample that was not observed at the HPLC purification step (UV monitoring), suggesting that the sample had undergone modification after isolation. The presence of this contaminant complicated the ^{13}C-NMR and ^{1}H-NMR analyses of the biosynthetic product because the spectra of the contaminant resembled those of 7β-hydroxytaxusin in many aspects. However, the two-dimensional NMR experiments clearly indicated the presence in the contaminant of a free hydroxyl at C9, and that the characteristic doublet of doublets had shifted downfield to δ 5.48 consistent with the chemical shift of a proton attached to a carbon (C7) now bearing an acetoxy group. These results were highly suggestive of intramolecular migration of

the acetyl group at C9 of the 7-hydroxytaxusin product to C7. Non-enzymatic shifts of this type have been observed previously in similarly functionalized taxoids [37]; the in vivo significance of such reactions is uncertain.

Sequence Analysis

The translated nucleotide sequence of clone F31 (GenBank accession no. AY307951, orf 1503 bp) encodes a 501-residue protein with a calculated molecular weight of 56,323. Analysis of the deduced sequence revealed several features that are characteristic of cytochrome P450 enzymes, such as an N-terminal membrane anchor, the oxygen binding domain, the reductase binding domain, the conserved PERF motif (aa 421-424), the highly conserved heme-binding motif with PFG element (aa 438-440), and the essential cysteine at position 446 [38]. Sequence alignment (Figure 3) of the taxoid 7β-hydroxylase from T. cuspidata with the previously acquired taxoid hydroxylases revealed significant identity and high similarity scores; for the 7β-hydroxylase versus the 5α-hydroxylase [27] (63% I, 82% S), the 10β-hydroxylase [26] (55% I, 75% S), the 13α-hydroxylase [28] (53% I, 72% S), and the 14β-hydroxylase [29] (53% I, 72% S). The 7β-hydroxylase is most similar in sequence to the 5α-hydroxylase which also hydroxylates in the taxane C-ring. However, the taxoid 5α-hydroxylase also bears high sequence identity to the taxoid 14β-hydroxylase which functionalizes the A-ring [29]. Thus, the correlation of sequence similarity with hydroxylation regiochemistry is inexact. In an EST library derived from Taxus cells induced for taxoid production with methyl jasmonate [25], clone F31 is the third most abundant cytochrome P450 transcript.

Characterization of the 7β-Hydroxylase

Because previous studies with taxoid hydroxylases have shown the Spodoptera fugiperda-baculovirus-based expression system to be superior to yeast for the characterization of Taxus cytochrome P450 enzymes (this highly efficient system coexpresses the Taxus NADPH cytochrome P450 reductase, permits ready isolation of catalytically functional microsomes, and is often less prone to interfering activities from the host), the 7β-hydroxylase clone F31 was transferred to the appropriate vector for this purpose [28]. Expression in these insect cells and microsome isolation by established protocols [27, 28] confirmed the efficient production of the recombinant cytochrome (~1 nmol/mL of resuspended microsomes) by CO-difference spectrometry [35] (Figure 4) and by standardized assay [27, 28] for the direct conversion of labeled taxusin to the 7β-hydroxy derivative (see Figure 5).

Using this microsomal system, the pH optimum for the 7β-hydroxylase was determined to be about pH 7.5, similar to other taxoid hydroxylases [28, 29]. Overall hydroxylase activity was slightly higher in Hepes buffer than in Tris-HCl; however, hydrolytic activity (enzymatic deacylation of both substrate and product) was also more prominent in Hepes. Therefore, Tris-HCl was selected as the buffer for kinetic assays but, even with this, it was still necessary to peracetylate the mixture prior to analysis to permit accurate quantification (Figure 5). By using this assay and Eadie-Hofstee plotting ($R^2 = 0.93$ for the line of best fit), the K_m value for (+)-taxusin with the recombinant microsomal 7β-hydroxylase was determined to be 7.6 ± 0.1 μM; this value is somewhat lower than those of other taxoid hydroxylases for their presumed natural substrates [27-29].

The occurrence in Taxus species of many 7β-oxygenated taxoids that vary in the oxygenation pattern and acyl substitutions on the taxane core [33], and the apparent abundance

of the F31 clone, suggest that the cytochrome P450 7β-hydroxylase could be a prominent catalyst in vivo that is promiscuous in substrate utilization and thus responsible for generating a range of 7β-hydroxy taxoids. To evaluate the specificity of the 7β-hydroxylase, a number of tritium labeled, simpler taxa-4(20),11(12)-diene derivatives (taxadiene, taxadien-5α-ol, taxadien-5α-yl acetate, taxadien-5α,13α-diol, taxadien-5α-acetoxy-10β-ol, and taxadien-5α-acetoxy-9α,10β,13α-triol (Fig. 6**E**)) were tested for catalytic conversion by feeding to the transformed yeast harboring clone F31, with [^{3}H-acetyl]taxusin as the positive control. None of these test substrates was detectably oxygenated in overnight assays under conditions where (+)-taxusin was nearly quantitatively converted to 7β-hydroxy taxusin. These results indicated that the 7β-hydroxylase is rather specific in being unable to utilize simple (relatively unsubstituted) taxoids as substrates, and they implied that the actual substrate for this enzyme is more highly substituted (hydroxylated and acylated), which is consistent with an intermediate of Taxol biosynthesis that resides somewhere in the middle of this extended pathway.

To assess this possibility, and obtain more information on the substrate selectivity of this enzyme, a range of more highly substituted taxoids was evaluated (see Figure 6). These compounds were not available in radiolabeled form, thereby eliminating the use of the simple in vivo catalytic assay in transformed yeast and necessitating the use of binding competence [39] (as an indirect measure of substrate selectivity) with Spodoptera microsomal preparations harboring the recombinant hydroxylase, again with (+)-taxusin as the positive control. The evaluation of these taxadien tetraol and pentaol acetates (Figure 6) showed taxusin (**A**) to exhibit the tightest binding (i.e., the lowest K_s value), followed in order by the pentaol pentaacetate (**G**), the tetraol triacetate (**B**) and the two tetraol diacetates (**C** and **D**), the tetraol monoacetate (**E**), the tetraol corresponding to taxusin (**F**), and finally the pentaol tetraacetate (**H**) with the weakest

binding affinity. Control microsomes (devoid of the clone F31 hydroxylase) did not appreciably bind these taxoid substrates.

Comparison of binding affinity between taxusin (**A**) and the corresponding tetraol triacetate (**B**) (180-fold difference), and between pentaol pentaacetate (**G**) and pentaol tetraacetate (**H**) (40-fold difference) indicated that the C5-acetate group is a very important binding determinant. Comparison of the K_s value of the tetraol monoacetate (**E**) with that of the corresponding tetraol (**F**) also supports the importance of the C5-acetate (i.e., two-fold difference in binding). Interestingly, comparison of monoacetate (**E**) with taxusin (**A**) reveals a 230-fold difference in binding, indicating that the C5-acetate alone is insufficient for tight binding and catalysis and suggesting that acylations elsewhere on the taxane core contribute to binding. This suggestion is also supported by comparing binding kinetics between the tetraol (**F**) and its various diacetylated (**C** and **D**) and triacetylated (**B**) derivatives (roughly two-fold difference). Additionally, comparison of the taxoids with either a free hydroxyl or acetate at C13 (cf. **B** & **D** and **C** & **E**) shows only minor differences in the K_s values, suggesting that acetylation at C13 is of little significance in binding. The addition of a C2-acetoxy group (cf. **A** and **G**) decreases binding by nearly 100-fold (perhaps due to steric interactions), although binding of the pentaacetate (**G**) is still more favorable (by a factor of two) than any of the partially acylated tetraols (cf. **G** to **B**, **C**, **D**, and **E**). Although productive binding is not necessarily indicative of functional oxygenation of the test substrate, the data do strongly suggest that the actual substrate for the 7β-hydroxylase is a well functionalized taxoid, possibly an acylated taxadien-tetraol bearing a 5α-acetoxy group, and that (+)-taxusin was a fortuitous choice as a surrogate for this mid-pathway intermediate. These data relating to the specificity of the cytochrome P450 taxoid 7β-hydroxylase (derived from both the catalytic and binding assays) also suggest that this

enzyme is relatively selective, which is not so unusual for a biosynthetic cytochrome P450 of this type. This conclusion implies that the large number of highly functionalized, naturally occurring 7β-hydroxy taxoid derivatives [33] do not arise via promiscuous substrate utilization by the 7β-hydroxylase but rather by the action of more promiscuous oxygenases and acyltransferases that lie downstream of the selective, and seemingly regiospecific, 7β-hydroxylation step.

Significance

A functional screen of Taxus cytochrome P450 clones using taxusin as a surrogate substrate yielded a taxoid 7β-hydroxylase that is similar in structure and properties to other taxoid hydroxylases, and that likely operates in the middle section of the Taxol biosynthetic pathway. Consistent with its placement on the pathway, selectivity studies suggest that the true substrate for this enzyme is a polyoxygenated and acylated taxoid bearing a 5α-acetoxy group. The 7β-hydroxy taxoids, and their C7 acetyl, benzoyl and xylosyl derivatives, are common metabolites of Taxus species [33]. Although the 7β-hydroxyl of Taxol is not essential for antimitotic activity [40], this functional group is the most reactive and accessible hydroxyl of the taxane core [5], and it has been exploited in the synthesis of biologically active photoaffinity probes [41-43], cleavable water soluble derivatives [44, 45], and the C7-methylthiomethyl analog as a highly effective "second generation" taxoid drug [46]. This cytochrome P450 7β-hydroxylase could prove useful in the improved production of Taxol in yew and for the preparation of other 7β-hydroxy taxoids as starting materials for subsequent modification at this position.

Experimental Procedures

Plant Material, Reagents and Substrates

The Taxus cell suspension cultures have been previously described [25]. The preparations of the racemic, simple taxoids taxa-4(5),11(12)-diene, taxa-4(20),11(12)-dien-5α-ol and taxa-4(20),11(12)-dien-5α-yl acetate, taxa-4(20),11(12)-dien-5α,13α-diol, and taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (all [20-^{3}H]-labeled at 2 Ci/mol) have been described [16, 17, 28, 47]. (+)-Taxusin was isolated from the heartwood of T. brevifolia [11], and taxa-4(20),11(12)-dien-2α,9α,10β,13α-tetraacetoxy-5α-ol was a gift from Dr. Tohru Horiguchi (Colorado State University, Fort Collins, CO).

[^{3}H-acetyl]Taxusin

(+)-Taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol, 75 μmol) was hydrolyzed to the tetraol in excess magnesium methoxide-methanol (8 mL) under reflux for 16 h, followed by dilution with brine and extraction into CH_2Cl_2. Solvent evaporation provided the tetraol (60% isolated yield), the identity of which was confirmed by comparison to the authentic standard. The tetraol (50 μmol) was reacetylated in CH_2Cl_2 (1 mL) containing dimethylaminopyridine (100 μmol), triethylamine (1 mmol) and [^{3}H]acetic anhydride (5 mCi, 100 μmol, NEN Life Sciences Products) by stirring for 16 h at room temp [48], followed by the addition of dimethylaminopyridine (50 μmol), triethylamine (200 μmol) and acetic anhydride (200 μmol) and stirring for another 20 h to complete the reaction. The reaction was quenched with saturated $NaHCO_3$, extracted with ethyl acetate (3 X 2 mL), and the pooled organic fraction was washed with brine and evaporated. The crude product was purified by preparative TLC

[silica gel developed twice with hexane:acetone (3:1, v/v)] to give (+)-[^{3}H-acetyl]taxusin (4.4 μmol, 146 Ci/mol, >95% purity by radio-HPLC).

Taxadien-5α-acetoxy-9α,10β,13α-triol

Because of the differing reactivities of the taxadien-tetraol hydroxyls [the 5α-hydroxyl is the most difficult to acylate, followed by 13α, 9α and 10β], protection was required to direct regiochemistry. The vicinal diol function was protected as the acetonide by stirring a solution of the tetraol (90 μmol) in 0.5 mL dimethylformamide containing p-toluene sulfonic acid (1.3 μmol) and 2,2-dimethoxy propane (2 mL) under reflux. The resulting acetonide was purified by silica gel flash column chromatography [hexane:acetone (3:1, v/v)] to give an isolated yield of 95% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.84 (s, CH_3), 0.97 (s, CH_3), 1.21 (dd, H14α, J = 3.9 Hz), 1.43 (d, CH_3 in acetonide, J = 0.6 Hz), 1.46 (d, CH_3 in acetonide, J = 0.3), 1.48 (s, CH_3), 1.67 (m, H2), 1.68 (m, H1), 1.74 (m, H7), 1.76 (m, H6), 2.08 (d, allylic-CH_3, J = 1.2 Hz), 2.85 (dt, H14β, J = 4.2, 9.8, 15.5 Hz), 3.16 (br t, H3, J = 2.1 Hz), 4.13 (d, H9, J = 9.3 Hz), 4.33 (t, H5, J = 2.5 Hz), 4.37 (br d, H13, J = 9.3 Hz), 4.70 (d, H20, J = 1.2 Hz), 4.90 (d, H10, J = 9.6 Hz), 5.07 (d, H20, J = 1.2 Hz).

To protect the 13α-hydroxyl, the TES ether was prepared from the acetonide (77 μmol) in 0.5 mL CH_2Cl_2 containing chlorotriethylsilane (13 μL), dimethylaminopyridine (50 μmol) and triethylamine (50 μL) by stirring at room temp for only 30 min to prevent silylation at the 5α-position. The reaction was quenched and extracted, and the product was purified by silica gel flash column chromatography as before to give an isolated yield of 34% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.23 (q, TES methyl, J = 8.0 Hz), 0.63 (t, TES methylene, J = 8.0 Hz), 0.68 (s, CH_3), 0.94 (dd, H14α, J = 2.0 Hz), 1.16 (s, CH_3 in acetonide),

1.20 (s, CH_3 in acetonide), 1.29 (s, CH_3), 1.48 (m, H2), 1.50 (m, H1, 1.60 (m, H7), 1.64 (m, H6), 1.85 (d, allylic-CH_3, J = 1.2), 2.56 (dt, H14β, J = 3.0, 9.2, 15.3 Hz), 3.47 (br t, H3, J = 4.8 Hz), 4.33 (d, H9, J = 9.6 Hz), 4.37 (t, H5, J = 2.6 Hz), 4.49 (br d, H13, J = 9.6 Hz), 4.66 (d, H20, J = 1.2 Hz), 5.04 (d, H20, J = 1.2 Hz), 5.23 (d, H10, J = 9.3 Hz). The C13-TES ether, 9,10-acetonide of the tetraol was then acetylated at C5 as above, and the product was purified by silica gel flash column chromatography [hexane:acetone (19:1, v/v)] to give an isolated yield of 36% at purity >95% as determined by ^{1}H-NMR. (300 MHz, $CDCl_3$) δ: 0.48 (q, TES methyl, J = 8.0), 0.88 (t, TES methylene, J = 8.0 Hz), 0.88 (s, CH_3), 1.13 (s, CH_3), 1.10 (dd, H14α, J = 8.0 Hz), 1.46 (s, CH_3 in acetonide), 1.50 (s, CH_3 in acetonide), 1.60 (s, CH_3), 1.70 (m, H2), 1.72 (m, H1), 1.76 (m, H7), 1.78 (m, H6), 2.02 (s, $COCH_3$), 2.02 (d, allylic-CH_3, J = 1.2 Hz), 2.40 (dt, H14β, J = 4.8, 9.6, 14.1 Hz), 2.88 (br t, H3), 4.32 (d, H9, J = 9.6 Hz), 4.62 (d, H20, J = 0.9 Hz), 4.77 (t, H13, J = 8.6 Hz), 5.00 (d, H10, J = 9.3 Hz), 5.07 (d, H20, J, = 0.9 Hz), 5.46 (t, H5, J = 2.7 Hz). The TES and acetonide groups were then removed in 0.5 N HCl by a published method [49], and the product was purified by silica gel TLC as above to give an isolated yield of 40% at purity >90% as determined by ^{1}H-NMR. (300 MHz, $CDCl_3$), δ: 0.90 (s, CH_3), 1.01 (s, CH_3), 1.25 (dd, H14α), 1.47 (s, CH_3), 1.68 (m, H2), 1.70 (m, H1), 1.72 (m, H7), 1.89 (m, H6), 2.06 (d, allylic-CH_3, J = 1.5 Hz), 2.10 (s, $COCH_3$), 2.86 (dt, H14β, J = 3.3, 9.9, 15.3 Hz), 3.01 (br t, H3, J = 3.8 Hz), 4.06 (d, H9, J = 9.3 Hz), 4.44 (dd, H13, J = 2.5 Hz), 4.82 (d, H10, J = 9.9 Hz), 4.86 (d, H20, J = 1.2 Hz), 5.19 (d, H20, J = 1.2 Hz), 5.35 (t, H5, J = 3 Hz).

Taxadien-2α,5α,9α,10β,13α-pentaol Pentaacetate

The pentaacetate (see Fig. 6**H**) was prepared from the corresponding tetraacetate (see Fig. **6G**), and purified by TLC as above, followed by HPLC [Metachem 5 μ Taxsil column (250 X 4.6 mm)

eluted with $CH_3CN:H_2O$ (1:4, v/v; 5 min hold) followed by a linear gradient (1 mL/min) to $CH_3CN:H_2O$ (4:1, v/v) with UV-monitoring] to give an isolated yield of 80% at purity >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.89 (s, CH_3), 1.14 (s, CH_3), 1.5 (dd, H14α, J = 7.2 Hz), 1.76 (s, CH_3), 1.73 (m, H7), 1.88 (m, H6), 1.94 (br d, H1, J = 9.6 Hz), 2.14 (d, allylic-CH_3, J = 1.2 Hz), 2.01 (s, $COCH_3$), 2.02 (s, $COCH_3$), 2.5 (s, $COCH_3$), 2.1 (s, $COCH_3$), 2.22 (s, $COCH_3$), 2.56 (dt, H14β, J = 4.2, 9.9, 15 Hz), 3.28 (d, H3, J = 6.6 Hz), 4.77 (d, H20), 5.29 (t, H5, J = 1.8 Hz), 5.34 (d, H20), 5.47 (dd, H2, J = 2.6 Hz), 5.91 (br t, H13, J = 8.9 Hz), 5.93 (d, H9, J = 10.5 Hz), 6.03 (d, H10, J = 10.8 Hz).

Taxadien-5α,13α-diacetoxy-9α,10β-diol

The 9,10-acetonide of taxusin tetraol prepared previously was acetylated at C5 and C13, deprotected, and purified as described above to give the diacetoxy diol at a purity of >95% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.91 (s, CH_3), 1.06 (dd, H14α, J = 8 Hz), 1.17 (s, CH_3), 1.52 (s, CH_3), 1.66 (m, H2), 1.72 (m, H7), 1.78 (m, H1), 1.82 (m, H6), 1.95 (d, allylic-CH_3, J = 1.8 Hz), 2.07 (s, $COCH_3$), 2.14 (s, $COCH_3$), 2.67 (dt, H14β, J = 4.5, 9.5, 14.8 Hz), 2.94 (br t, H3, J = 4.8 Hz), 4.14 (d, H9, J= 9.6 Hz), 4.80 (d, H10, J = 9.6 Hz), 4.82 (d, H20, J = 1.5 Hz), 5.19 (d, H20, J = 1.5 Hz), 5.35 (t, H5, J = 3 Hz), 5.54 (br t, H13, J = 8.7 Hz).

Taxadien-9α,10β,13α-triacetoxy-5α-ol

Taxusin tetraol was partially acetylated as before but without protecting groups, and the reaction was monitored by analytical TLC and quenched after the completion of triacetate formation (1h). The triacetoxy monool product was purified by flash chromatography as above to give an isolated yield of 60% at purity >90% as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.71 (s,

CH_3), 0.99 (s, CH_3), 1.13 (dd, H14α, J = 4.9 Hz), 1.57 (s, CH_3), 1.72 (m, H7), 1.75 (m, H1), 1.78 (m, H2), 1.82 (m, H6), 2.10 (d, allylic-CH_3, J = 1.5 Hz), 2.01 (s, $COCH_3$), 2.05 (s, $COCH_3$), 2.07 (s, $COCH_3$), 2.79 (dt, H14β, J = 4.8, 10.1, 15.3 Hz), 3.29 (d, H3, J = 3.9 Hz), 4.29 (t, H5, J = 3 Hz), 4.75 (d, H20, J = 1.5 Hz), 5.12 (d, H20, J = 1.5 Hz), 5.71 (dd, H13, J = 3.9 Hz), 5.76 (d, H9, J = 10.5 Hz), 6.09 (d, H10, J = 10.5 Hz).

Taxadien-9α,10β-diacetoxy-5α,13α-diol

Taxusin tetraol was regioselectively acetylated with recombinant taxoid 10-O-acetyltransferase [22] which is capable of acylating hydroxyls at both C9 and C10 positions. The isolated product was purified by HPLC as above to give the diacetoxy diol at >95% purity as determined by ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.70 (s, CH_3), 0.90 (s, CH_3), 1.20 (dd, H14α, J = 3.9 Hz), 1.50 (s, CH_3), 1.70 (m, H1), 1.70 (m, H6), 1.74 (m, H2), 1.92 (m, H7), 2.22 (d, allylic-CH_3, J = 1.2 Hz), 2.00 (s, $COCH_3$), 2.04 (s, $COCH_3$), 2.81 (dt, H14β, J = 3.3, 9.1, 15.6 Hz), 3.26 (d, H3, J = 4.5 Hz), 4.31 (t, H5, J = 2.7 Hz), 4.33 (dd, H13, J = 3 Hz), 4.71 (d, H20, J = 1.2 Hz), 5.07 (d, H20, J = 1.2 Hz), 5.71 (d, H9, J = 10.5 Hz), 6.07 (d, H10, J = 10.2 Hz).

Taxus Cell Microsome Preparation and Assay

Taxus cuspidata suspension cell cultures (cell line P93AF) were induced with methyl jasmonate, the frozen cells (40 g) were extracted (1 mM benzamidine was substituted for phenylmethylsulfonyl fluoride in the buffer) and microsomes were prepared by established protocols [16, 17]. [H^3-acetyl]Taxusin (10^6 dpm, 10 Ci/mol) was added to a 10 mL screw-capped tube and the assay was preformed as previously described [17]. The diethyl ether extract was evaporated, and the residue was dissolved in 2 mL of pentane:ether (1:1, v/v) and loaded

onto a small column of silica gel (Mallinckrodt SilicAR 60 Å) which was washed with 6 mL of the same solvent to remove residual substrate, followed by elution with 8 mL diethyl ether to obtain the more polar products. The latter fraction was evaporated, and the residue was dissolved in CH_3CN and separated by radio-HPLC [Metachem Taxsil column developed with CH_3CN:H_2O (1:4, v/v; 5 min hold) followed by a linear gradient (1 mL/min) from 20% to 80% CH_3CN with monitoring using a Packard A-100 flow-through radio-detector].

Cytochrome P450 Expression and In Vivo Assay in Yeast

Procedures for the functional expression of Taxus cytochrome P450 cDNA clones in S. cerevisiae WAT11 cells [34] and the in vivo assay have been previously described [26, 29]. For the purpose of product identification, a 250 mL expression culture (A_{600} ~0.8) was divided into 10 mL aliquots from which the transformed cells were harvested by centrifugation and each resuspended in 3 mL of induction medium to which 100 µM (+)-taxusin (in DMSO) was added. Following incubation for 16 h at 30°C with shaking, each tube was treated in a sonication bath (10 min), saturated with NaCl, and extracted with hexane (3 X 2 mL). The pooled extracts were evaporated, and the residue was dissolved in pentane:ether (1:1, v/v) and loaded onto and eluted from a silica gel column with the same solvent to remove residual substrate. The presumptive hydroxyl taxusin product was then eluted from the column with ether, the solvent concentrated, and the material purified by TLC [silica gel developed once with hexane:acetone (19:1, v/v) and twice with hexane:acetone (3:1, v/v)], eluted from the gel with acetone, concentrated and dissolved in CH_3CN and further purified by HPLC [Alltech Adsorbosphere HS C18, 5 µ column (250 X 4.6 mm) with an isocratic gradient of 40% CH_3CN in H_2O. Fractions containing the

product were combined, concentrated, and extracted with pentane to provide pure material for spectrometric analysis.

Spectrometric Analyses

To simplify MS analysis of the putative hydroxyl taxusin (that contained partially deacetylated material from host-derived esterases), the mixture was peracetylated and purified by reverse-phase HPLC as described above. LC-MS (atmospheric pressure chemical ionization, APCI) analysis was performed on a Hewlett-Packard (Agilent) series 1100 HPLC with model 1946A mass detector. The product was resolved on a Supelcosil Discovery HS-F5 column (250 x 4.6 mm) with a Metachem Metaguard HS-H5 guard column. The column was developed with CH_3CN:H_2O (1:19) at 5% to 100% CH_3CN over a 50 min linear gradient (1 ml/min). The MS method has been previously described [32].

Coupled capillary GC-MS analyses were performed on a Hewlett-Packard 6890 MSD system (electron impact at 70 eV ionizing voltage) using a Phenomenex ZB-5 column (30 m x 0.25 mm, 5% phenyl polysiloxane) and cool on-column injection, with a previously described method [17].

For NMR analysis of the purified product, the material was repeatedly dissolved in and evaporated to dryness from $CDCl_3$ and then dissolved in 0.5 mL $CDCl_3$. ^{1}H-NMR was performed on a 300 MHz Varian Mercury NMR spectrometer, and ^{13}C-NMR was performed on a 600 MHz Bruker NMR spectrometer. The nuclear Overhauser effect difference (NOE-DIF) experiment, two-dimensional NOE spectroscopy (NOESY) and two-dimensional homonuclear total correlation spectroscopy (TOCSY) were performed on the Varian 300 MHz instrument.

Cytochrome P450 Expression in Sf9 Cells

Heterologous expression of Taxus cytochrome P450 cDNA clones in Spodoptera furgiperda (Sf9) cells that co-express the Taxus NADPH:cytochrome P450 reductase was carried out as previously described [28], except that the baculovirus infection period was extended to 45 min and harvest was extended to 60 h post-infection. Microsome preparation was also as before [28] but, in this case, the lysis buffer contained 20 mM Hepes (pH 7.5), 5 mM DTT, 10 mM lidocaine, 10 mM procaine and 1 mM benzamidine, and the final resuspension buffer was 25 mM Tris (pH 7.5) containing 20% (v/v) glycerol. The concentration of microsomal cytochrome P450 was determined by CO-difference spectroscopy [35], and the assay for oxygenation of taxusin (and other taxoids) was as before (with 100 pmol of cytochrome P450 in a 0.5 mL assay volume) but the NADPH concentration was reduced to 0.5 mM and a regenerating system (2 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase) was added. Incubation was for 16 h at 31ºC in the dark, with product extraction and purification as before. Control experiments were identically carried out but with a β-glucuronidase gene replacing the cytochrome P450 insert.

Characterization of the 7β-Hydroxylase

The pH optimum of the recombinant taxoid 7β-hydroxylase (cytochrome P450 clone F31 expressed in Spodoptera) was determined in Mopso (pH 6.5-7.5), Hepes (pH 7.0-8.0), Tris (pH 6.5-8.0) and phosphate (pH 5.0-9.5) buffers over intervals of 0.5 pH units. The K_m value for (+)-taxusin (substrate concentration range of 5 to 300 μM) was determined by Eadie-Hofstee plotting using Microsoft Excel software. To simplify the radio-HPLC assay described above, the products were peracetylated prior to analysis. Conversion was determined by integration of the

pentaacetate product radio-peak relative to that of the substrate, and the reported values are the means ± SD of three independent replicates.

Substrate binding assays [39] were performed with a Perkin-Elmer Lambda 18 spectrophotometer using established protocols [17] and Spodoptera microsomes containing the 7β-hydroxylase. Double-reciprocal plotting of the difference between the maximum (390 nm) and minimum (420 nm) absorbance for each substrate concentration was used to determine the binding constant (K_S).

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Table 1.

Complete ^{1}H and ^{13}C NMR Assignments for the Biosynthetic Product Derived from Taxusin by the Recombinant Cytochrome P450 Expressed from Clone F31

Position number	α-Proton δ (ppm)	β-Proton δ (ppm)	Carbon δ (ppm)
1		1.85 (m)	35.66
2	1.72 (m)	1.77 (m)	31.42
3	2.83 (br d)		40.21
4			146.89
5		5.39 (br t)	75.15
6	2.09 (m)	1.63 (m)	36.49
7	4.34 (dd)		70.65
8			46.72
9		6.05 (d)	79.36
10	6.23 (d)		71.34
11			134.57
12			137.63
13		5.92 (t)	70.37
14	1.05 (dd)	2.66 (dt)	27.37
15			39.50
16	1.14 (s, exo)		27.37
17	1.63 (s, endo)		31.22
18	2.1 (s)		12.27
19		0.80 (s)	15.05
20	4.93 (d, exo)		115.38
	5.27 (d, endo)		
OAc	2.1 (s)		20.81
	2.07 (s)		21.01
	2.02 (s)		21.46
	2.16 (s)		21.77
			168.90
			169.68
			169.77
			170.41

For position numbering, see Figure 1.

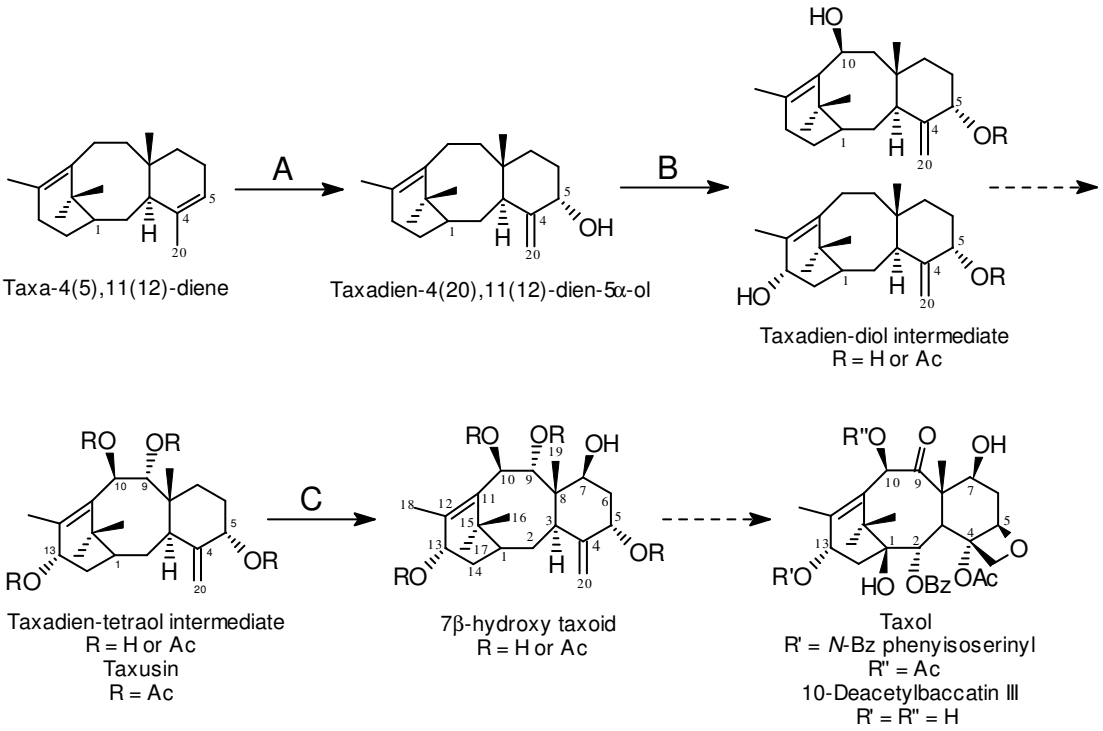

Figure 1. Outline of the Taxol Biosynthetic Pathway

Hydroxylation of the committed precursor taxa-4(5),11(12)-diene to the monool by the 5α-hydroxylase (A), hydroxylation to the level of a diol by the 10β-hydroxylase or 13α-hydroxylase (B), and hydroxylation of a tetraol derivative by the 7β-hydroxylase (C) are illustrated. Broken arrows indicate multiple steps, several of which remain undefined.

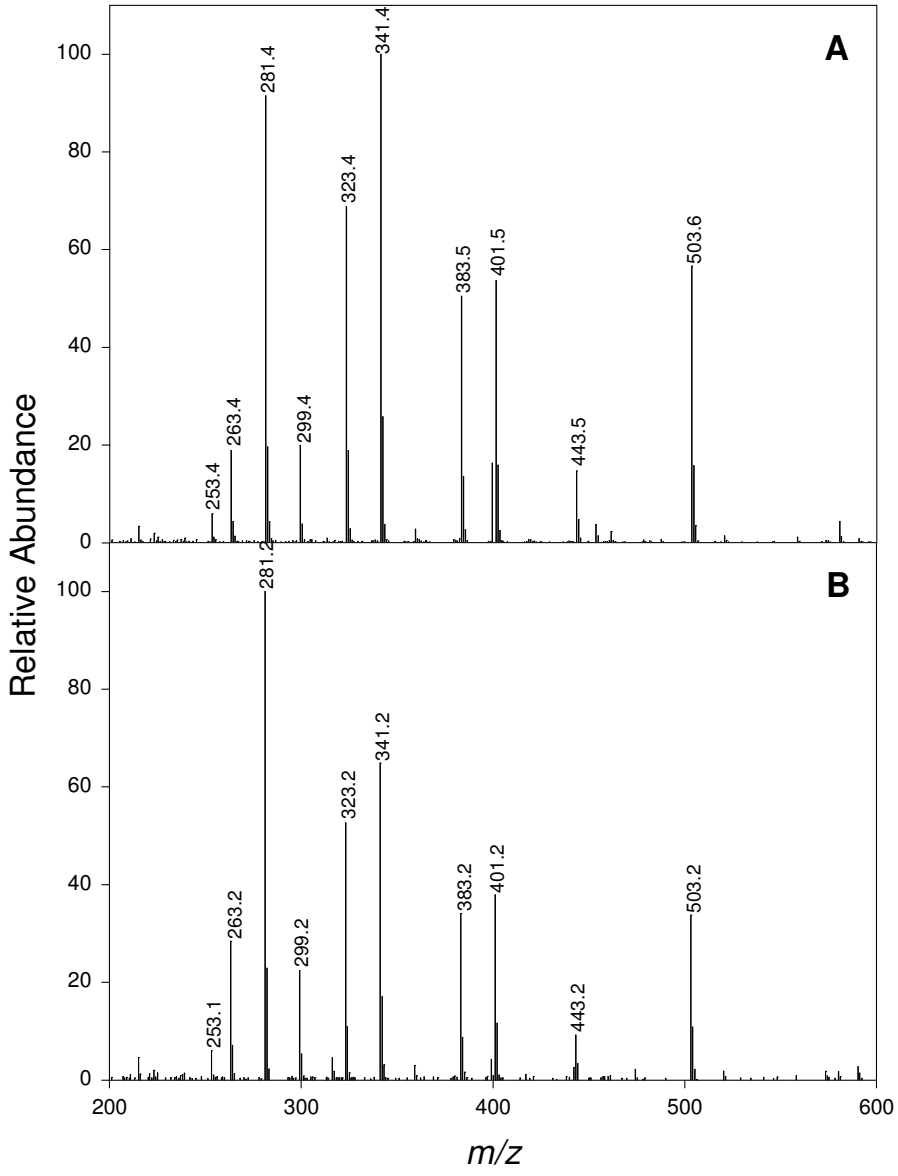

Figure 2. LC-MS of the Biosynthetic Product Derived from (+)-Taxusin

The mass spectra (APCI) of the presumptive hydroxytaxusin (following peracetylation) derived from taxusin by Taxus cell microsomes (A) and by recombinant cytochrome P450 clone F31 expressed in yeast (B) are illustrated; the LC retention times of both products (A and B) were also identical (33.6 min).

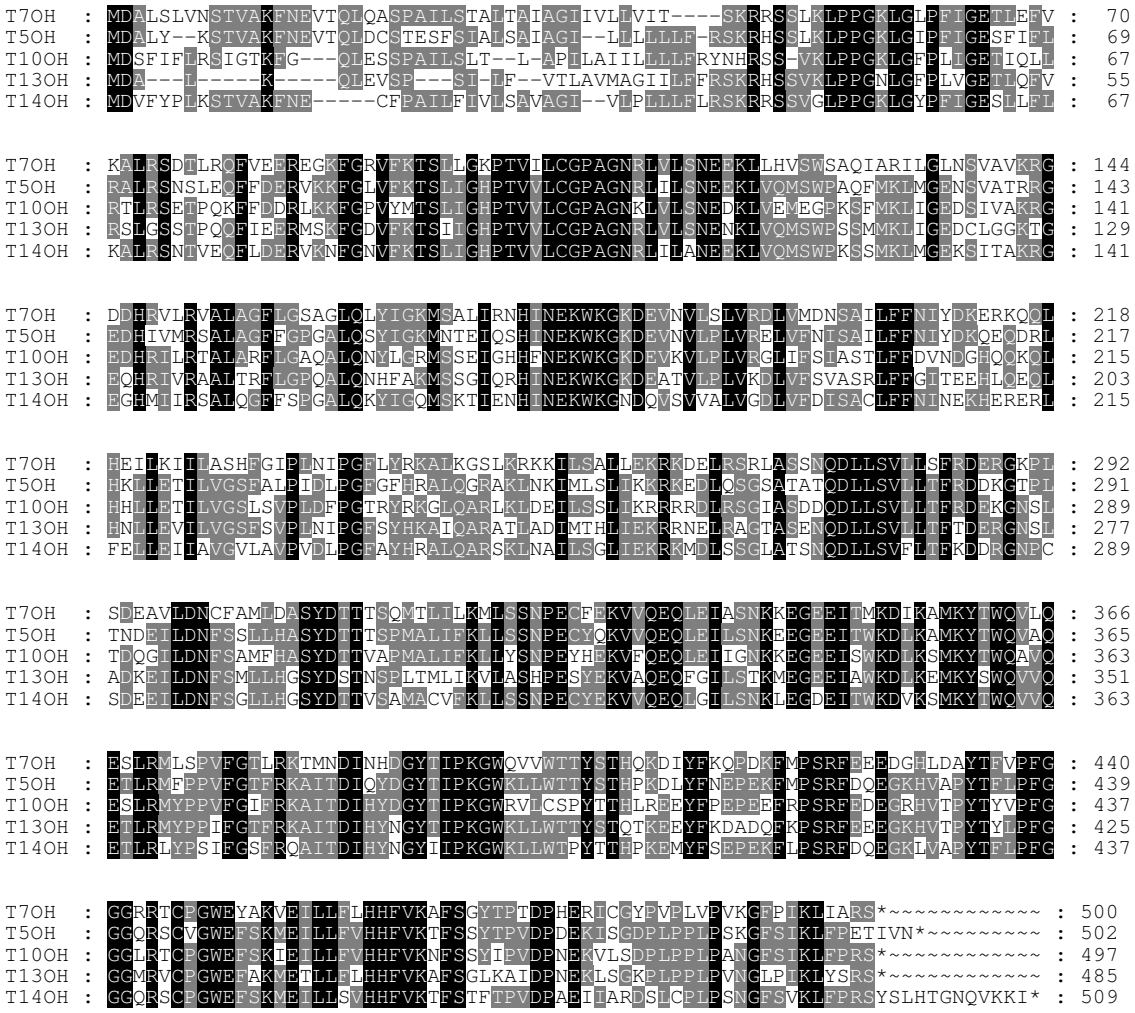

Figure 3. Deduced Sequence Alignment of Taxoid Hydroxylases

Alignments of cytochrome P450 taxoid 7β-hydroxylase (T7H) with taxoid 5α-hydroxylase (T5H), 10β-hydroxylase (T10H), 13α-hydroxylase (T13H) and 14β-hydroxylase (T14H) are illustrated. Black boxes indicate identical residues; grey boxes indicate identical residues for at least three of the sequences.

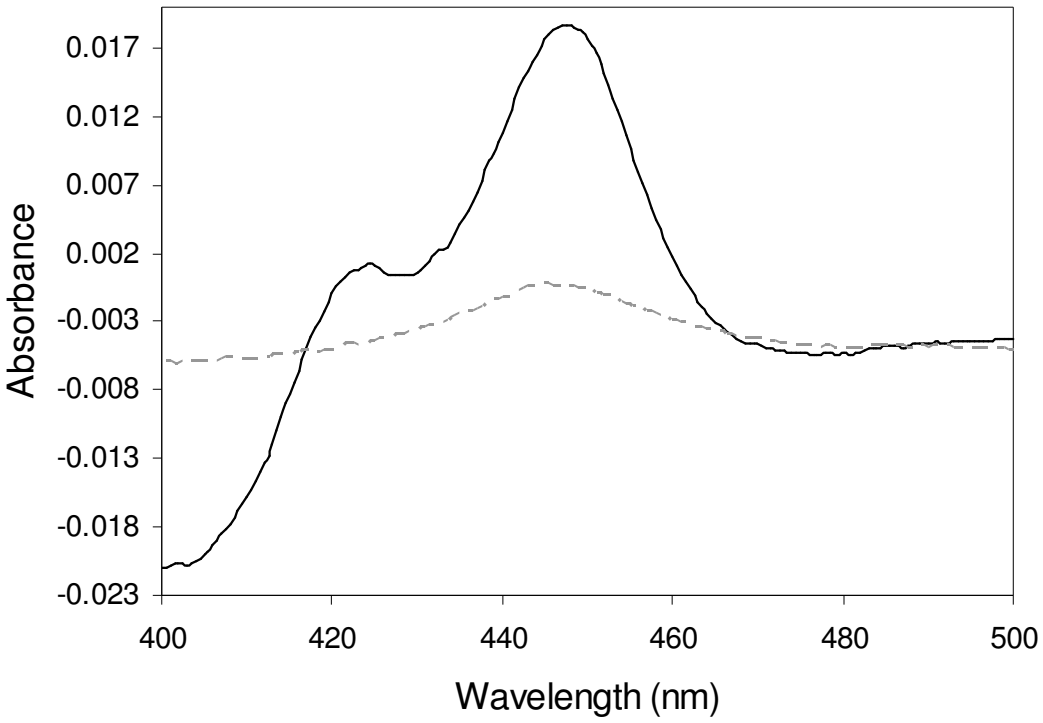

Figure 4. Reduced CO-Difference Spectrum of the Recombinant Hydroxylase

The spectrum was recorded using a microsomal preparation from Sf9 cells which express recombinant cytochrome P450 clone F31 (solid line); the dotted line is the negative control spectrum identically obtained using microsomal preparations from Sf9 cells which express recombinant β-glucuronidase.

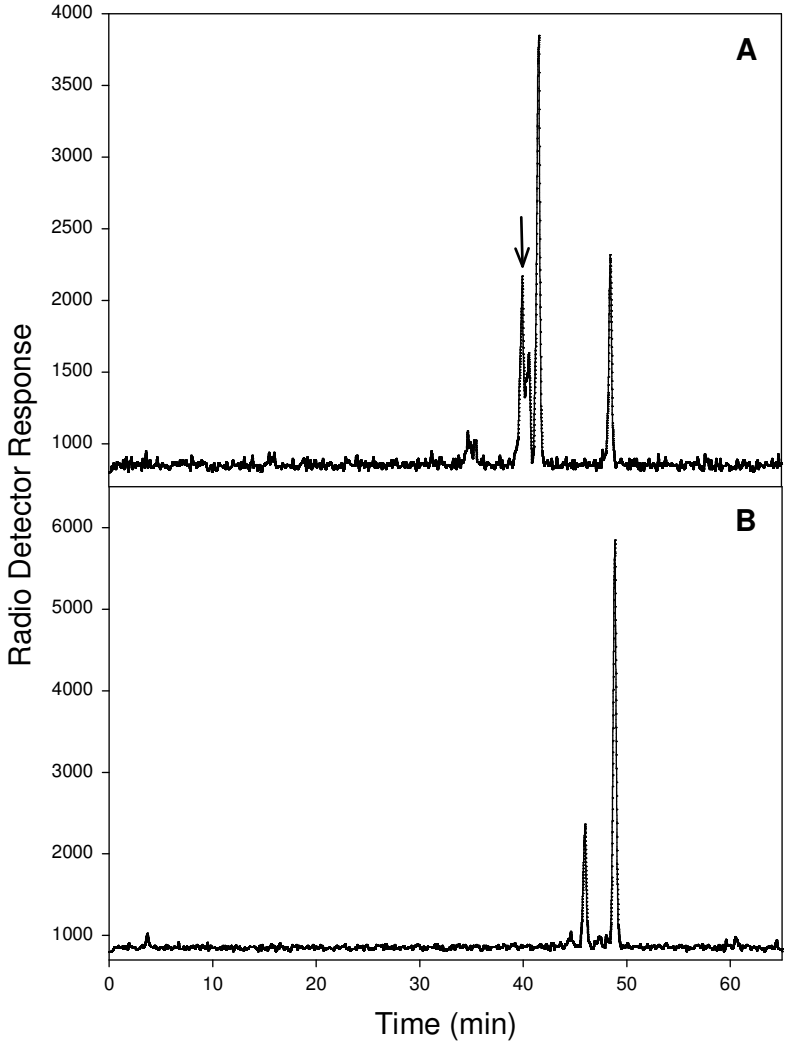

Figure 5. Radio-HPLC Analysis of the Products Derived from [^{3}H]Taxusin

Panel A illustrates the mixture of products generated from [^{3}H-acetyl]taxusin by the microsomal cytochrome P450 hydroxylase expressed from clone F31 in Sf9 cells, with the substrate at 49 min, the hydroxytaxusin product at 40 min (arrow), and a mixture of partial hydrolysis products with retentions between 40 and 41 min. Panel B illustrates the separation of the same mixture following chemical peracetylation, with the substrate (tetraacetate) eluting at 49 min and the derived pentaacetate product eluting at 46 min.

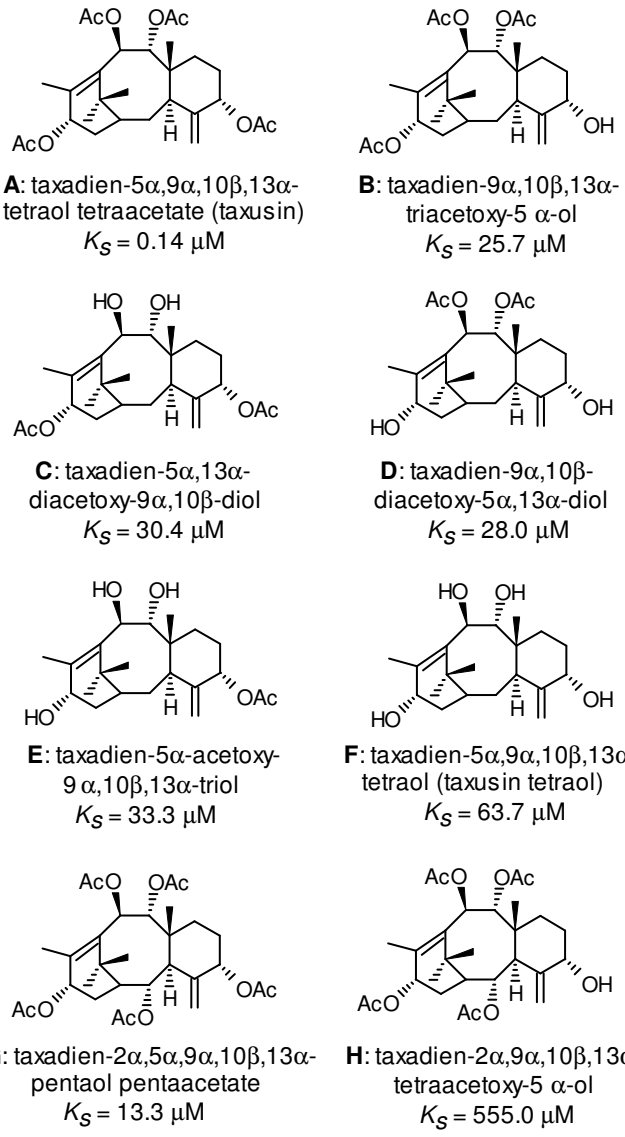

Figure 6. Taxoid Substrates Used in Binding Studies with the 7β-Hydroxylase

The binding constant ($\underline{K_S}$) is shown below each polyfunctionalized taxoid.

CHAPTER THREE

Molecular cloning and characterization of a cytochrome P450 taxoid 2α-hydroxylase involved in Taxol biosynthesis

Abstract

The Taxol biosynthetic pathway, arising from the primary isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate in yew (Taxus), consists of approximately twenty steps, at least nine of which are thought to be cytochrome P450-mediated oxygenations. Several oxygenases involved in the early hydroxylation steps of the pathway have been identified and the corresponding genes have been cloned; however, defining the enzymes and their genes responsible for oxygenations in the central portion of the pathway is more difficult because neither the exact sequence of reactions nor the relevant intermediates are known. A surrogate substrate, (+)-taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate), that was previously employed in the isolation of a taxoid 7β-hydroxylase, was used here to functionally screen a family of cytochrome P450 oxygenases originating from a Taxus cell EST library. This in vivo screen in yeast led to the identification of a 1488 bp cDNA clone (encoding a 495 residue protein) that was capable of producing 2α-hydroxytaxusin from taxusin with a K_M value of 10.5 ± 2.7 μM and k_{cat} of about 0.05 s^{-1} for this surrogate substrate. This structurally typical cytochrome P450 resembles most closely the previously isolated taxoid 7β-hydroxylase, which also uses taxusin as a substrate, and both 2α- and 7β-hydroxylases are capable of the reciprocal conversion of their respective pentaol tetraacetate products to the common hexaol tetraacetate. This C2-hydroxylase would appear to mediate the mid-pathway functionalization of the C2-position of the taxane core that ultimately bears a benzoyl group as an important Taxol pharmacophore. Overexpression of this cytochrome P450 taxoid 2α-hydroxylase in Taxus cells may improve Taxol yields and could prove useful in the production of other 2α-hydroxy taxoids as starting materials for subsequent acylation at this position.

Introduction

Taxol (paclitaxel) is one of the most effective antineoplastic drugs currently available, and is used to treat a range of cancers, either alone [1-3] or in combination with other chemotherapeutic agents [4-7]. Taxol (Fig. 1) is a highly functionalized taxane diterpenoid (taxoid) that acts, unlike most spindle poisons, by promoting tubulin polymerization (and preventing depolymerization) and ultimately leading to cell death [8]. The structural elements (pharmacophores) responsible for the cytotoxicity of Taxol, in addition to the rigid taxane skeleton, include the oxetane ring (D-ring), the N-benzoylphenylisoserine side chain appended to C13 of the A-ring, the benzoate group at C2 of the B-ring, and the acetate function at C4 of the C-ring [9]. Because total synthesis of Taxol is impractical [10-13], the increased demand for the drug prompted a shift from destructive isolation from the original, low-yielding source (the bark of the Pacific yew, Taxus brevifolia) to semisynthetic approaches [14]. These involve synthetic side chain attachment to more readily available, advanced taxoids, such as 10-deacetylbaccatin III (Fig. 1), that can be isolated from the needles of various Taxus species as a renewable resource. Because supply of this anticancer drug for the foreseeable future will continue to rely on yew species or, potentially, cell cultures derived therefrom [15, 16], it is important to understand the biosynthesis of Taxol and its underlying molecular genetics, with the view to improving production yields.

The Taxol biosynthetic pathway is considered to require 19 enzymatic steps from the universal diterpenoid precursor geranylgeranyl diphosphate [17] which is cyclized, in the committed step, to taxa-4(5),11(12)-diene [18]. This parental olefin (Fig. 1) is then functionalized by a series of eight cytochrome P450-mediated oxygenations, three CoA-dependent acylations and several other transformations en route to baccatin III (Fig. 1), to which

the side chain at C13 is appended to afford Taxol (Fig. 1). The proposed order of hydroxylations on the taxane core, based on an assessment of the oxygenation patterns of the naturally occurring taxoids [19] and biochemical studies on early pathway steps [20, 21], is thought to begin with C5 and C10, then C2, C9 and C13 (exact order uncertain), followed by C7, and finally C1. A family of related cytochrome P450 cDNA clones, isolated by differential display of mRNA-RT-PCR[1] [22], homology-based cloning [23], and an EST project [24] and functionally screened in yeast or Spodoptera with available test substrates [20, 25], has yielded genes encoding the taxoid 5α-, 10β-, and 13α-hydroxylases responsible for early pathway steps [22, 23, 26].

The mid-pathway hydroxylations have been more difficult to approach because of the uncertainties about the exact sequence of these steps and intermediates, and the lack of readily available test substrates. For this reason, more accessible 'surrogate' substrates have been prepared for test of function of the remaining cytochrome P450 clones. For example, (+)-taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate, Fig. 2), an abundant yew heartwood metabolite [18, 27] that is unlikely on the route to Taxol [19], was prepared in radiolabeled form and used to screen clones for the C1, C2, and C7 hydroxylases, an approach that recently yielded the taxoid 7β-hydroxylase [28]. The large number of differentially-oxygenated taxoids isolated to date [29] and the relatively small number of candidate cytochrome P450 genes thus far identified that seemingly account for this diversity [24] suggested that substrate selectivity of the recombinant hydroxylases might be reasonably broad, and thus encouraged the continued testing of the surrogate substrate taxusin as a cloning tool. In this paper, we describe the isolation and preliminary characterization of a new cDNA encoding a taxoid 2α-hydroxylase that is capable of converting (+)-taxusin to the 2α-hydroxy derivative (Fig. 2).

Materials and Methods

Enzymes, substrates, and reagents

Unless otherwise indicated, enzymes and vectors were obtained from Invitrogen (Carlsbad, CA) and chemicals were purchased from Sigma (St. Louis, MO). The preparations of racemic, tritium-labeled taxa-4(5),11(12)-diene, taxa-4(20),11(12)-dien-5α-ol, and taxadien-5α-yl acetate (each at 2 Ci/mol), as well as (+)-taxusin (10 Ci/mol), (+)-taxadien-5α-acetoxy-10β-ol (11 Ci/mol), and (+)-taxadien-5α-acetoxy-9α,10β,13α-triol (53 Ci/mol), have been described [20, 25, 28, 30]. Taxadien-5α,10β-diol (11 Ci/mol) was generated from the corresponding diol monoacetate precursor by deacetylation using magnesium methoxide [28].

Generation of full-length clones by 5′-RACE

An EST library [24] prepared from T. cuspidata cells elicited with methyl jasmonate for increased Taxol production [31] yielded several new, putative cytochrome P450 sequences closely related (~70% S) to those of taxoid hydroxylases previously isolated by other cloning methods [22, 23]. Both full-length and 5′-truncated partial genes (in the original pBluescript vector) were fully sequenced, and full-length versions of the partial genes were obtained by 5′-RACE (Invitrogen, version 2.0) from total RNA isolated from T. canadensis [32].

For the partial cDNA designated 3156, three gene specific primers were designed: 5′-ACTTGCCATGTGTATCTCAT-3′ (GSP1), 5′-TCATAGCATTCTGGATGGTC-3′ (GSP2), and 5′-TCGTAGGAGCCATCAATGAGCGC-3′ (GSP3). The 5′-RACE abridged anchor primer (AAP) sequence was 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′.

The product of first strand synthesis, using 5 μg RNA and 2 pmol of primer GSP1, was purified (Edge Biosystems Spin column) and employed for 3′-poly-C addition and second strand amplification with the AAP forward and GSP1 reverse primers (94°C for 1 min, then 40 cycles at 94°C for 40 s, 53°C for 1 min, and 72°C for 2 min, with final extension at 72°C for 15 min). The PCR product of appropriate size was gel purified and extracted (Qiagen gel-extraction kit), and employed in a second PCR reaction (same conditions) but substituting reverse primer GSP2 for GSP1. The ds cDNA product was purified as before and used in a third amplification with reverse primer GSP3 and forward primer AAP. This final PCR yielded a product of appropriate size (~1000 bp) that was excised and gel purified as before, and cloned into pCR 2.1-TOPO and transformed into Escherichia coli TOP 10 cells for kanamycin selection and PCR-based insert screening using M13R and M13F primers according to the manufacturer's protocol (Invitrogen pYES2.1 TOPO TA Expression Kit).

Positive transformants were grown for plasmid preparation (Qiagen mini-prep kit), sequencing and analysis (GCG, Wisconsin Package Version 10.0, Madison, WI; using the PILEUP program) to compare the newly acquired sequence with related cytochrome P450 genes and the original partial 3156 gene sequence. Following verification, forward primer (3156_FOR: 5′-ATGGACGCCATGGATCTCACAG-3′) and reverse primer (3156_REV: 5′-GGATCGAGAAATAAGTTTAATAGG-3′) were designed to isolate the full-length gene (but from which the stop codon was deleted to permit epitope tagging) from the original T. canadensis transcript pool. Reverse transcription was carried out as before except that primer 3156_REV was used in place of GSP1. The full-length version was amplified from the reverse transcribed product using the 3156_FOR and 3156_REV primers, and Pfu polymerase which required modification of the PCR method (extension temperature 68°C for 3 min and the final

extension at 68°C for 15 min). To add A-overhangs to the amplicon, Taq polymerase (1 U) was added at the end of the reaction and the temperature was maintained at 72°C for another 10 min. The resulting amplicon (~1500 bp) was excised, gel purified, cloned into pYES 2.1 TOPO, and used to transform E. coli TOP 10 F′ cells, which were selected on ampicillin. Positive transformants were grown for plasmid preparation to permit determination of proper insert orientation (T7-20mer and 3156_REV primers) and sequence verification.

Yeast expression and immunoblot analysis

pYES plasmids containing full-length 3156 cDNA inserts were used to transform Saccharomyces cerevisiae WAT11 cells [33] by the lithium acetate method [34] but with steelhead sperm DNA as carrier. Yeast cultures were grown in 10 ml SGAI media until stationary phase, transferred to 200 ml SGAI media and grown until stationary phase, and the cells then harvested by centrifugation (2000 x g 10 min) and used to inoculate 1 L of YPAL induction media [33]. This induced culture was grown (275 rpm, 30°C, ~12 hours) until A_{600} reached ~1.5, and the cells were harvested as before for microsome preparation by the glass bead method [33]. Microsomal proteins were solubilized in 6 M urea, quantified by the Bradford method [35] and separated (50 µg protein) by SDS-PAGE (10% denaturing gel) in preparation for immunoblotting [36]. This approach to monitoring expression takes advantage of deletion of the stop codon in the original construct to generate a C-terminally appended polyhistidine (His_6) tag encoded by the pYES 2.1 TOPO vector; previous evidence has shown that such epitope tagging does not influence the activity or expression efficiency of other cytochrome P450 taxoid hydroxylases [23, 37]. Monoclonal PentaHis-specific antibody (Qiagen, Valencia, CA), along

with alkaline phosphatase-conjugated rabbit anti-mouse IgG as secondary antibody (Jackson ImmunoResearch, West Grove, PA), were employed for detection.

For in vivo feeding, as a means to functionally screen cytochrome P450 clones in intact yeast, the cells were grown as previously described [22, 37], aliquoted and resuspended in fresh induction media, and 250,000 dpm of each radiolabeled taxoid substrate (in 10 μL DMSO) was individually added to the corresponding assay. The resuspended cells were incubated (300 rpm) for 16 h at 30°C, then saturated with NaCl, treated in a sonication bath, and extracted thoroughly with ethyl acetate (3 x 2 ml), followed by centrifugation to separate the phases. The pooled organic extracts were evaporated and the residue dissolved in CH_3CN for radio-HPLC analysis.

Product analysis

Because esterase activity in the yeast host is capable of hydrolyzing taxusin and its hydroxylated product(s), half of each sample from above was peracetylated with acetic anhydride prior to radio-HLPC separation under previously described conditions [28] to convert hydrolyzed substrate to taxusin and any hydroxylated taxusin to the corresponding pentaacetate, thereby minimizing ambiguity in the analysis and permitting direct comparison to taxadien-pentaol pentaacetate standards. The product mixtures generated both by the recombinant enzyme and by Taxus microsomes, both before and after peracetylation, were partially purified by HPLC and analyzed by LC-MS (APCI) using a previously reported method [38].

To generate sufficient product for structural identification by NMR, the above described assay was scaled up 50-fold, and the product was extracted and purified as before. The resulting hydroxytaxusin (~1.2 mg) was repeatedly dissolved and evaporated to dryness from $CDCl_3$ and finally dissolved in 300 μL $CDCl_3$. ^{1}H-NMR, NOESY, and COSY experiments were performed

on a Varian Mercury 300 MHz instrument. ^{13}C-NMR and HMQC experiments were performed on a Bruker DRX 600 MHz instrument.

Reciprocal conversions by taxoid hydroxylases

To determine if the 2α-hydroxytaxusin and 7β-hydroxytaxusin products of the respective taxoid 2α- and 7β-hydroxylases could be reciprocally utilized as substrates by these enzymes, their biosynthetic products formed from labeled taxusin were purified by HPLC as above. These hydroxytaxusins (5 x 10^5 dpm) were then diluted with authentic material (to 10 μM in the assay) and fed to transformed yeast harboring the 7β-hydroxylase or 2α-hydroxylase according to the standard protocol. The resulting hexaol tetraacetate products were extracted and separated by radio-HPLC, and the partially purified materials were subjected to LC-MS (APCI) analysis [38]. Both products were also peracetylated to permit LC-MS comparison of properties to an authentic sample of taxa-4(20),11(12)-dien-2α,5α,7β,9α,10β,13α-hexaol hexaacetate obtained previously as a Taxus cell culture metabolite [38]

Enzyme Characterization

Yeast microsomes containing the recombinant hydroxylase were prepared by literature procedure [33], except that the TESB buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.6 M sorbitol) used contained 5 mM DTT and 1 mM benzamidine and the TEG buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20% glycerol) contained 5 mM DTT. The microsomal pellet was resuspended in TEG buffer (~3 mL/1 L initial culture) and the cytochrome P450 concentration (~0.3 μmol/mL) was determined by CO-difference spectroscopy [39]. The pH optimum of the recombinant microsomal hydroxylase was determined in phosphate (pH 5.0-9.5), Hepes (pH 7.0-

8.0), and Tris-HCl (pH 6.5-8.0) buffers over intervals of 0.5 pH units. The assays contained 50 pmol of recombinant cytochrome P450, 50 μM [^{3}H]taxusin (added in 3 μL DMSO), 200 nM NADPH, 2.5 mM each of FAD and FMN in 300 μL of Tris-HCl buffer (pH 7.8); the assays were run at 31°C for 20 min in the dark. Kinetic analyses were performed in Tris-HCl, pH 7.8, with substrate concentrations ranging from 5 to 200 μM. The K_M value for (+)-taxusin was determined by Eadie-Hofstee plotting using Microsoft Excel software, and is reported as the mean ± standard error of triplicate assays; substrate concentration was averaged to account for conversion in the assay. Reaction products were partitioned between brine and ethyl acetate (2 x 2 ml), the organic solvent was evaporated, and the residue was dissolved in 100 μL CH_3CN for radio-HPLC analysis, both before and after peracetylation with acetic anhydride as previously described [28].

Results and Discussion

Cloning of a (+)-taxusin hydroxylase

In a previous attempt to search for mid-pathway hydroxylases of Taxol biosynthesis, the potential surrogate substrate (+)-taxusin was employed with Taxus microsomes in a preliminary experiment to assess the feasibility of this approach for defining these transformations [28]. This experiment revealed by radio-HPLC analysis (Fig. 3) that several products were generated from taxusin in microsomal preparations; one of these was subsequently identified as 7β-hydroxytaxusin and ultimately led to the cloning of a cDNA encoding the corresponding taxoid 7β-hydroxylase [28]. Another product was slightly more polar than the first but nevertheless possessed a retention time by HPLC that was consistent with that of a hydroxylated taxusin.

Although insufficient material was available to permit NMR-based structural elucidation of this second product, this observation encouraged further screening of the recombinant cytochrome P450 clones with this surrogate substrate to search for the corresponding putative hydroxylase.

Because cytochrome P450 clones obtained by differential display [22] and by homology-based methods [23] had been previously screened with taxusin without result, we turned our attention to the T. cuspidata EST library from which several new full-length cytochrome P450 clones and partial sequences had been obtained. Following acquisition of additional sequence information by 5′-RACE where necessary, full-length forms were amplified using T. canadensis mRNA as template, and these clones were installed in S. cerevisiae WAT11 cells for expression [33]. The WAT11 strain is constructed to allow galactose-inducible coexpression of the NADPH cytochrome P450 reductase from Arabidopsis [33] and permits a very simple preliminary screen by feeding the intact cells with taxoid substrates [22]. However, previous evidence [37] had shown that cytochrome P450 expression in this yeast strain can be difficult to measure by classical CO-difference spectra methods [39]. Therefore, as an alternative, a C-terminally fused His_6-tag (encoded by the pYES 2.1 TOPO vector) was appended to the cytochrome P450 to allow monitoring of expression by immunoblotting. This procedure does not compromise the activity or expression efficiency of other cytochrome P450 taxoid hydroxylases [23, 37].

Yeast harboring the target clones, and verified to express the corresponding recombinant cytochrome P450 (~55 KDa) by immunoblotting, were fed labeled taxusin, and the overnight incubations were extracted and the products analyzed by radio-HPLC. One clone, designated 3156 from the induced Taxus cell EST library, was capable of the nearly quantitative conversion of taxusin to a more polar product with a retention time identical to that of the previously noted

product generated from taxusin by Taxus cell microsomes (Fig. 3). None of the other taxoid test substrates was detectably utilized by cytochrome P450 clone 3156 expressed in yeast.

Product identification

LC-MS analysis of the product derived from taxusin by clone 3156 revealed diagnostic fragment ions that were consistent with a hydroxytaxusin (Fig. 4A). The parent ion (P^+) was not observed at m/z 521 [taxusin MW = 504 plus O (mass 16) + H^+] but diagnostic fragment ions were observed at m/z 461 (P^+-CH_3COOH), 401 (P^+-$2CH_3COOH$), 359 (P^+-$2CH_3COOH$-CH_3CO), 341 (P^+-$3CH_3COOH$), 299 (P^+-$3CH_3COOH$-CH_3CO), and 281 (P^+-$4CH_3COOH$). LC-MS analysis of the product derived from taxusin by Taxus microsomes also revealed a product with the identical mass spectrum (Fig. 4B) as well as the same retention time (30.1 min). Although authentic hydroxytaxusin standards are not readily available, acetylation of the above products derived from Taxus microsomes and by the recombinant enzyme followed by HPLC purification and LC-MS analysis of the peracetylated material, yielded the same product (in both cases) which has a retention time (33.7 min) and mass spectrum identical to that of authentic taxa-4(20),11(12)-dien-2α,5α,9α,10β,13α-pentaol pentaacetate (Fig. 4C & D) [28]. Like the underivatized product, the parent ion of the pentaacetate was again not observed at m/z 563 [acetoxytaxusin (MW = 562) + H^+]; however, diagnostic fragment ions were observed at m/z 503 (P^+-CH_3COOH), 443 (P^+-$2CH_3COOH$), 401 (P^+-$2CH_3COOH$-CH_3CO), 383 (P^+-$3CH_3COOH$), 341 (P^+-$3CH_3COOH$-CH_3CO), 323 (P^+-$4CH_3COOH$), 299 (P^+-$3CH_3COOH$-$2CH_3CO$), 281 (P^+-$4CH_3COOH$-CH_3CO), and 263 (P^+-$5CH_3COOH$). These results strongly suggested that cytochrome P450 clone 3156, as well as Taxus microsomes, catalyzed the conversion of (+)-taxusin to the 2α-hydroxy derivative.

To confirm this identification, the transformed yeast incubations with taxusin were scaled-up 50-fold, and the presumptive hydroxytaxusin product was purified as before to yield about 1.2 mg of material (>95% pure by HPLC) for NMR analysis (Table 1). ^{13}C-NMR assignments were aided by comparison to those of taxusin [40] and by HMQC analysis. Comparison of the ^{1}H-NMR spectrum of taxusin with that of the putative 2α-hydroxytaxusin revealed a doublet of doublets at 4.22 ppm in the latter, consistent with the chemical shift of a proton attached to a carbon bearing a hydroxyl group. The only possible protons that could exhibit this chemical shift are at either C2 or C7; however, the proton at C7 bearing a hydroxyl had already been assigned a different chemical shift [28], suggesting that the observed doublet of doublets was due to the C2 proton of 2-hydroxytaxusin . 2D-NMR COSY and NOESY analyses were performed to verify the regio- and stereochemistry of this hydroxytaxusin product. COSY data showed strong correlations with H3 and moderate correlations with H1, further supporting the proposed C2-hydroxyl function. NOESY analysis revealed strong correlations with H16 (methyl), moderate correlations with H19 (methyl), and weak correlations with H1. Models indicated that only the C2 β-proton is capable of exhibiting these correlations, whereas the α-proton would exhibit correlations with H20 endo and H14α protons, which were not observed. These results confirm the identification of the biosynthetic product as 2α-hydroxytaxusin and, thus, that clone 3156 encodes a taxoid 2α-hydroxylase.

Sequence analysis

Sequence information from the T. cuspidata induced cell EST library [24] along with that obtained by 5′-RACE using a similar message pool obtained from T. canadensis induced cells [32] permitted the amplification of full length hydroxylase clones using the T. canadensis mRNA

pool and RT-PCR. A number of these clones were fully sequenced to reveal several variants that differed slightly from the original sequences obtained. Sequence variants have been previously observed in the T. cuspidata EST library [24] for many of the genes involved in taxoid metabolism, and these, and their homologues from other Taxus species [23], tend to be very similar (>95% S). In vivo assay with taxusin of each of these full-length clones in yeast showed a roughly seven-fold difference in apparent 2α-hydroxylase activity, and the most active of these, designated clone 3156.5, showed the following substitutions relative to the original sequence information: S56P, Q159K, I196M, N215K, A222P, L254Q, S259R, L263V, S278A, F279Y, K280E, V313M, and T440K (see Fig. 5). The immunoblotting procedure was not sufficiently sensitive to distinguish small differences in expression levels and it is assumed that differences in apparent activity levels observed reflect inherent properties of the corresponding recombinant taxoid 2α-hydroxylase. Thus, the N215K substitution occurs in the predicted second substrate recognition site [41], and may be anticipated to influence binding and/or catalysis. Also, the A222P substitution might influence secondary structure because of its predicted placement between the F- and G-helices [42]. Because of its seemingly greater encoded hydroxylase activity, clone 3156.5 was employed for product generation (above) and for all subsequent studies.

Clone 3156.5 (orf 1488 bp) codes for a 495-residue protein with a calculated molecular weight of 55,471 and exhibits other characteristic sequence elements of cytochrome P450 enzymes [43], including a typical N-terminal membrane anchor, the oxygen binding domain, the reductase binding domain, the conserved PERF motif (amino acids 416-419), the highly conserved PFG element (amino acids 433-435), the EXXR salt bridge (amino acids 362-365), and the essential cysteine at position 441. Pair-wise comparison of the taxoid 2α-hydroxylase

with previously isolated cytochrome P450 taxoid hydroxylases revealed significant sequence similarity and identity: with taxoid 7β-hydroxylase, 81% S, 65% I; taxoid 5α-hydroxylase, 78% S, 62% I; taxoid 10β-hydroxylase, 72% S, 54% I; taxoid 13α-hydroxylase, 71% S, 53% I; and taxoid 14β-hydroxylase, 73% S, 56% I. The taxoid 2α-hydroxylase resembles most closely the taxoid 7β-hydroxylase which also utilizes the same (+)-taxusin substrate (see also Fig. 5). In the T. cuspidata EST library, the taxoid 2α-hydroxylase is moderately abundant at 0.7‰, and is the sixth most abundant of the seven cytochrome P450 taxoid hydroxylases thus far defined.

Characterization of the taxoid 2α-hydroxylase

In previous instances, instability of recombinant cytochrome P450 taxoid hydroxylases in microsomes prepared from the yeast host has forced the utilization of the baculovirus-Spodoptera fugiperda expression system for detailed characterization of these enzymes [26]. In the present case, however, yeast microsomal preparations harboring the recombinant taxoid 2α-hydroxylase yielded readily measurable oxygenase activity and CO-difference spectra, and thus were used for enzyme characterization. The pH optimum was determined to be about pH 7.8 in Tris-HCl buffer, which is typical for this enzyme type. The K_M value for (+)-taxusin with the recombinant microsomal 2α-hydroxylase was determined to be 10.5 ± 2.7 μM by Eadie-Hofstee plotting (R^2 = 0.87 for the line of best fit). This value is slightly lower than those of other cytochrome P450 taxoid hydroxylases for their presumed natural substrates, and so indicates the fortuitous choice of taxusin as a surrogate substrate for examining the mid-pathway hydroxylation steps of Taxol biosynthesis. A k_{cat} of approximately 0.05 s^{-1} was determined for this enzyme with (+)-taxusin as substrate.

Reciprocal feeding studies with 2α- and 7β-hydroxylases

Since both the taxoid 2α-hydroxylase and the taxoid 7β-hydroxylase [28] can employ taxusin as substrate and the two are quite similar in primary structure (Fig. 5), reciprocal feeding studies were performed to determine if the corresponding hydroxytaxusin products of each hydroxylase could be converted by the other respective enzyme to a common hexaol tetraacetate (i.e., 2α,7β-dihydroxytaxusin). In vivo assays were initially conducted by mixing transformed yeast cells individually harboring one or the other hydroxylase and feeding labeled taxusin. Radio-HPLC analysis of the products of this incubation revealed the presence of 2α-hydroxytaxusin and 7β-hydroxytaxusin but no dihydroxytaxusin, suggesting either that the proposed conversions were not possible or that the hydroxytaxusins formed remained largely within the yeast cells and were not freely diffusible as might have been anticipated based on the efficient uptake and conversion of taxusin itself.

To examine these possibilities in greater detail, the hydroxytaxusin products were individually purified, and 7β-hydroxytaxusin was administered to yeast cells harboring the 2α-hydroxylase while 2α-hydroxytaxusin was fed to yeast cells harboring the 7β-hydroxylase. In this instance, radio-HPLC analyses revealed the presence of a new, more polar product in each case (the presumptive hexaol tetraacetate; see Fig. 2), each having a common retention time before (28 min) and after (42 min) peracetylation. MS analysis of this presumptive dihydroxytaxusin revealed the absence of the predicted parent ion at m/z 537 [taxusin MW = 504 plus 2 O (mass 32) + H^+] but diagnostic fragment ions consistent with a hexaol tetraacetate were noted at m/z 477 (P^+-CH_3COOH), 459 (P^+-CH_3COOH-H_2O), 417 (P^+-$2CH_3COOH$), 399 (P^+-$2CH_3COOH$-H_2O), 375 (P^+-$2CH_3COOH$-CH_3CO), 357 (P^+-$3CH_3COOH$), 339 (P^+-$3CH_3COOH$-

H_2O), 315 (P^+-3CH_3COOH-CH_3CO), 297 (P^+-4CH_3COOH), and 279 (P^+-4CH_3COOH-H_2O). Although a reference standard for the predicted product, 2α,7β-dihydroxytaxusin, was not available, peracetylation of the products allowed direct comparison to authentic taxa-4(20),11(12)-dien-2α,5α,7β,9α,10β,13α-hexaol hexaacetate, a metabolite previously isolated from Taxus cell suspension cultures [38], and demonstration of the identical HPLC retention time (42 min) and mass spectrum to confirm the identification. The parent ion for this hexaacetate was not observed at m/z 621 [diacetoxytaxusin (MW = 620) + H^+]; however, a diagnostic adduct and characteristic fragment ions were observed at m/z 638 (P^++NH_4^+), 561 (P^+-CH_3COOH), 501 (P^+-2CH_3COOH), 459 (P^+-2CH_3COOH-CH_3CO), 441 (P^+-3CH_3COOH), 417 (P^+-2CH_3COOH-2CH_3CO), 399 (P^+-3CH_3COOH-CH_3CO), 381 (P^+-4CH_3COOH), 357 (P^+-3CH_3COOH-2CH_3CO), 339 (P^+-4CH_3COOH-CH_3CO), 321 (P^+-5CH_3COOH), 297 (P^+-4CH_3COOH-2CH_3CO), 279 (P^+-5CH_3COOH-CH_3CO), and 261 (P^+-6CH_3COOH).

Both enzymes catalyzed the conversion of the respective hydroxytaxusin to 2α,7β-dihydroxytaxusin at lower efficiencies than the conversion of taxusin to the corresponding 2α- or 7β-hydroxytaxusin; the rates of conversion of hydroxytaxusin were 30% and 5% for the taxoid 2α-hydroxylase and taxoid 7β-hydroxylase, respectively, of their rates of conversion of taxusin. Based on apparent substrate utilization efficiencies, these results may suggest the order of hydroxylation in vivo (i.e., 7β-hydroxylation followed by 2α-hydroxylation); however, such judgment may be premature because of experimental limitations of the feeding studies (i.e., differences in expression levels and precursor uptake). Interestingly, incubation of Taxus cell microsomes with taxusin, followed by peracetylation to collapse the many hydrolyzed metabolites to either starting material or derived products and LC-MS analysis, revealed not only the presence of pentaol pentaacetates but also the hexaol hexaacetate (i.e., the third product of

microsomal metabolism described above). These results (data not shown) indicate that Taxus microsomes possess sufficient activity of the two cytochrome P450 oxygenases to permit sequential hydroxylation of taxusin to the level of a hexaol tetraacetate.

Conclusions

The length and complexity of the Taxol biosynthetic pathway, coupled to the difficulty in obtaining relevant taxoid intermediates, has led to the use of (+)-taxusin (a presumed dead-end metabolite of yew heartwood) as a surrogate substrate to examine mid-pathway oxygenation steps en route to Taxol. (+)-Taxusin proved to be a very productive surrogate substrate in allowing the functional screening of cytochrome P450 clones to identify cDNAs encoding the taxoid 7β-hydroxylase [28] and the taxoid 2α-hydroxylase described in this paper. The 2α-hydroxylase is similar in sequence and kinetics to other cytochrome P450 taxoid hydroxylases. The work described here indicates that the taxoid 2α-hydroxylase and the taxoid 7β-hydroxylase, both of which efficiently utilize taxusin, can reciprocally convert the corresponding 2α- and 7β-hydroxytaxusin products of the respective reactions to the common 2α,7β-dihydroxytaxusin, although none of the structurally simpler taxoid substrates was utilized by either hydroxylase. The demonstration that these recombinant enzymes are capable of utilizing more than one surrogate taxoid substrate suggests similar plasticity in substrate utilization in planta and implies that the pathway to Taxol may not be linear but rather a network of anastomosing routes with perhaps several common nodes on the way. A similar conclusion was reached previously based on substrate selectivity studies with the taxoid 10β- and 13α-hydroxylases [26]. Given the very large number (>350) of structurally defined taxoids [29], and that even multiple pathways from taxadiene to Taxol likely involve only 19 basic biochemical steps, there must also exist several

side routes and diversions responsible for the formation of taxines, taxinines, taxuyunnanines, and other "dead end" metabolites. The substrate selectivities of the taxoid hydroxylases and acyltransferases almost certainly play a central role in the "metabolic dispersion" of so many products from the common precursor taxadiene.

The C2 hydroxyl, located on the B-ring southern hemisphere of the taxane skeleton, bears a benzoyl moiety in Taxol, and this functional grouping is important for tubulin binding and promoting cytotoxicity [44]. However, the benzoyl group, itself, is not essential for activity, and it can be substituted with other aroyl or acyl groups to afford 'second generation' drugs with greater potency than Taxol [45-47]. The newly described taxoid 2α-hydroxylase has helped define an additional step in the biosynthesis of Taxol, and manipulation of this gene could prove useful for increasing the production yields of Taxol in yew and in the generation of alternate 2α-hydroxytaxoids as starting materials for subsequent acyl and aroyl addition.

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Footnotes

1 Abbreviations used: APCI, atmospheric pressure chemical ionization; COSY, correlated spectroscopy; DTT, dithiothreitol; EST, expressed sequence tag; GCG, Genetics Computer Group; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris-(hydroxymethyl)-aminomethane.

Table 1.

Complete ^{1}H and ^{13}C NMR assignments for the biosynthetic product derived from taxusin by the recombinant cytochrome P450 expressed from clone 3156

Position number	Proton δ (ppm)	Carbon δ (ppm)
1	2.12 (m)	51.34
2	4.22 (dd) J = 2.3, 7.1	69.95
3	3.13 (d) J = 6.6	45.99
4		143.50
5	5.28 (br t) J = 2.7	78.32
6α	1.7 (m)	28.72
6β	1.85 (m)	
7	1.6 (m)	27.56
8		44.39
9	5.86 (d) J = 10.5	76.35
10	6.02 (d) J = 10.8	72.41
11		133.03
12		136.65
13	5.96 (t) J = 8.9	70.55
14α	1.34 (m)	28.00
14β	2.57 (dt) J = 4.2, 9.6	
15		37.35
16	1.69 (s)	27.19
17	1.17 (s)	31.62
18	2.1 (d) J = 1.2	14.84
19	0.91 (s)	15.05
20	5.43 (t) J = 1.5	119.18
	5.45 (s)	
OAc	2.00 (s)	20.85
	2.05 (s)	21.02
	2.09 (s)	21.46
	2.21 (s)	21.82
		169.87
		170.00
		170.27
		170.39

For position numbering, see Figure 1.

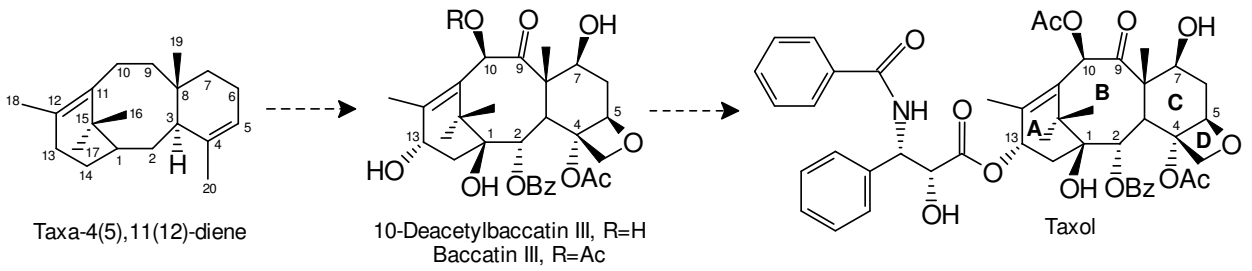


Figure 1. The conversion of taxadiene to baccatin III requires eight oxygenations on the taxane core, and one in the C13-side chain of Taxol.

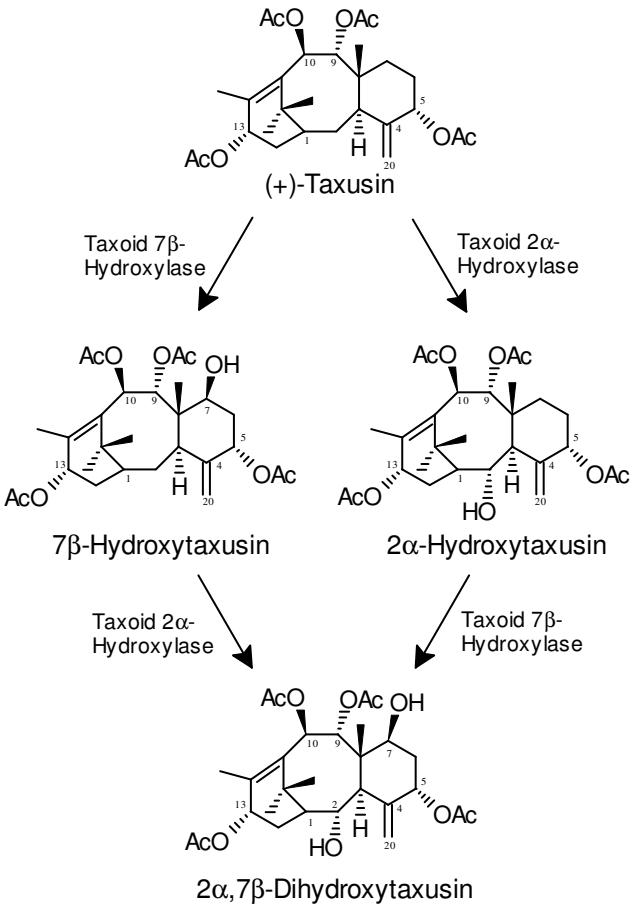


Figure 2. Conversions mediated by taxoid 7β-hydroxylase [28] and the taxoid 2α-hydroxylase described in this paper.

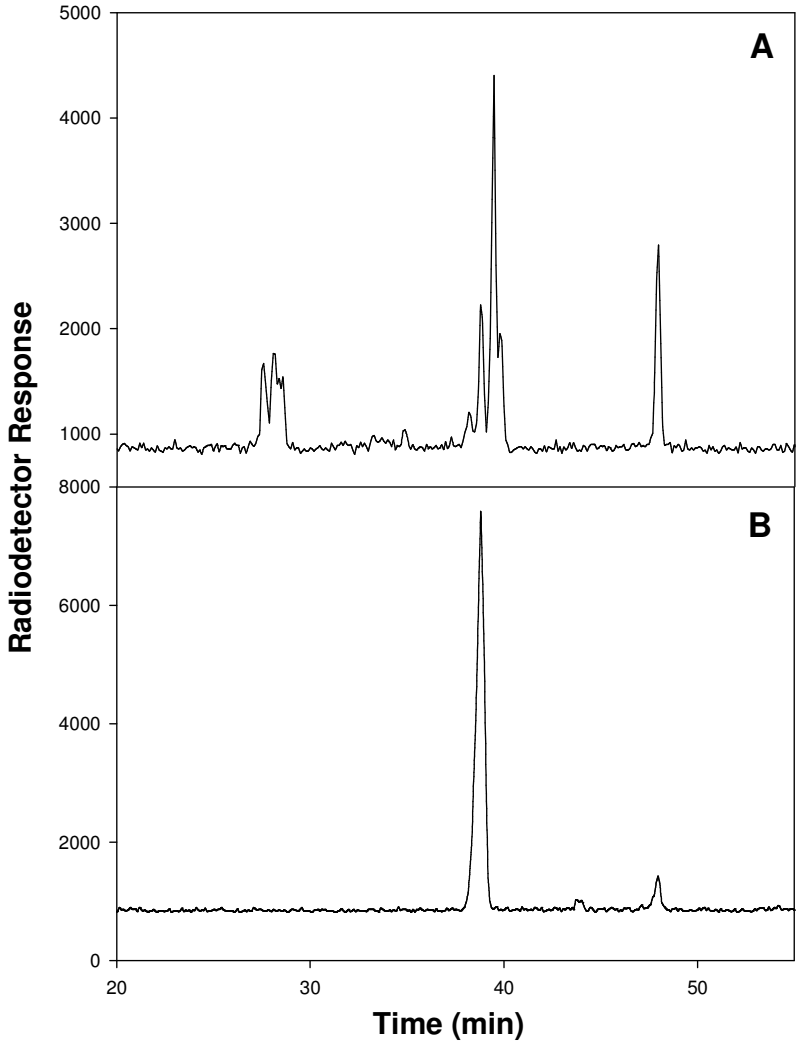
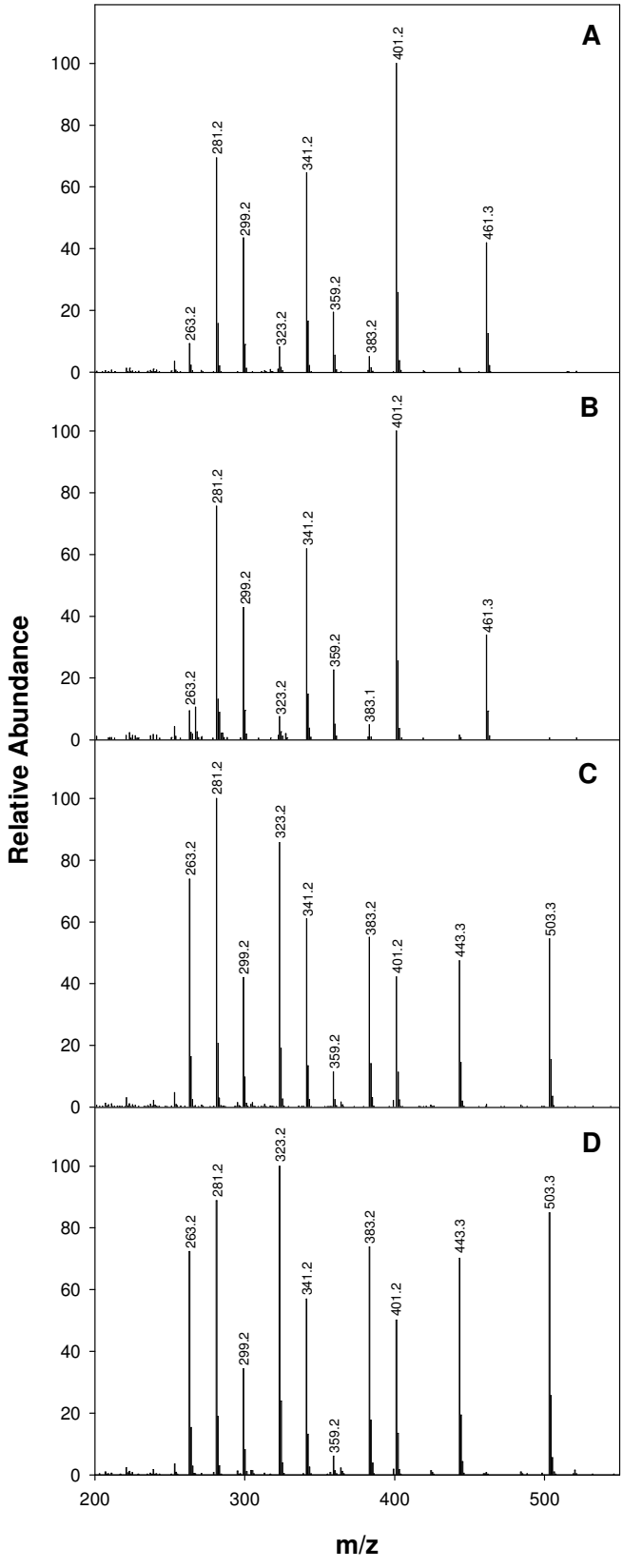


Figure 3. Reversed-phase radio-HPLC analysis of (A) the products (both biosynthetic and hydrolytic) of Taxus cuspidata microsomes incubated with (+)-[^{3}H-acetyl]taxusin and of (B) the biosynthetic product (R_t = 38.8 min) derived from exogenous (+)-[^{3}H-acetyl]taxusin (R_t = 48.2 min) administered to yeast cells harboring cytochrome P450 cDNA clone 3156.5. The product purified by this means was identified by LC-MS and NMR methods as 2α-hydroxytaxusin. The retention time of 7β-hydroxytaxusin is 39.9 min.

Figure 4. LC-MS analysis of the purified biosynthetic products ($\underline{R_t}$ = 30.1 min) derived from (+)-taxusin by yeast cells harboring cytochrome P450 cDNA clone 3156.5 (A) and by T. cuspidata suspension cell microsomes (B). Also provided are the APCI-MS of the peracetylated product (C) from (A) above, and of authentic taxadien-2α,5α,9α,10β,13α-pentaol pentaacetate (D); both products (C & D) have identical LC retention times (33.7 min).


A
B
C
D
Relative Abundance
m/z
100
80
60
40
20
0
200
300
400
500
263.2
281.2
299.2
323.2
341.2
359.2
383.2
401.2
443.3
461.3
503.3
383.1

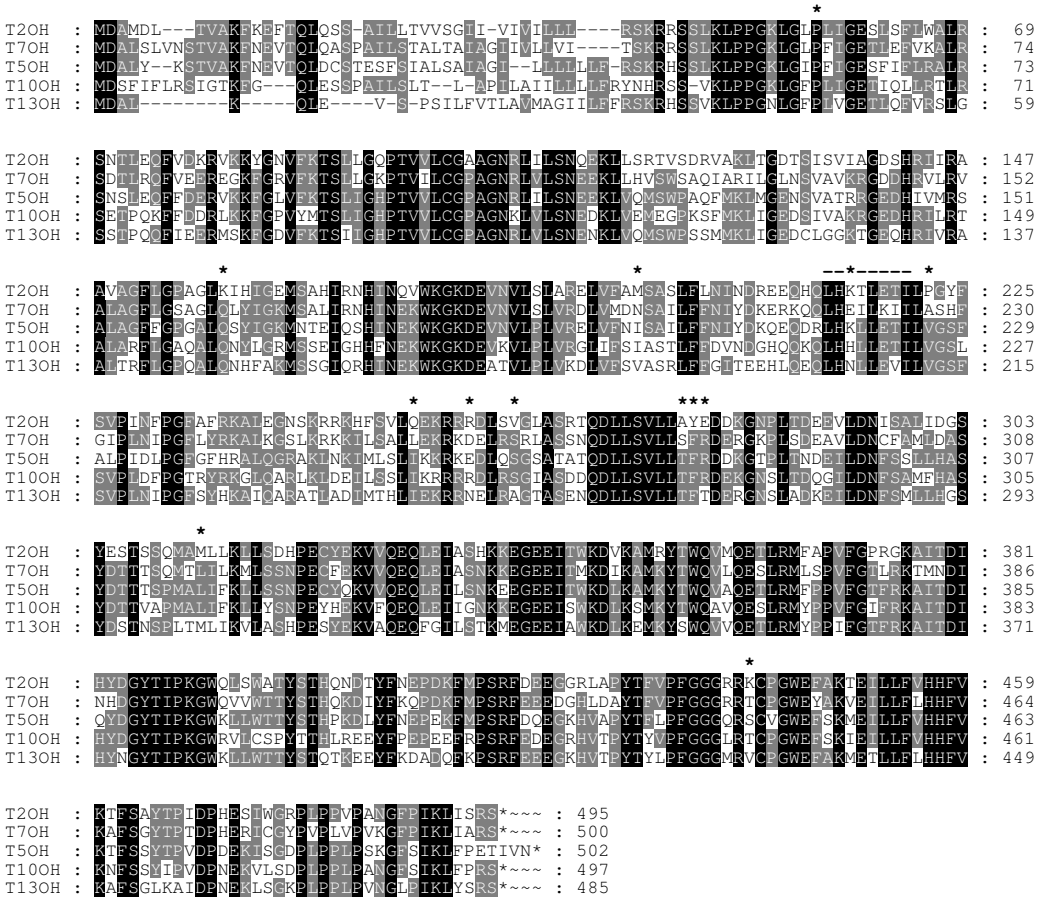


Figure 5. Deduced amino acid sequence alignment of the taxoid 2α-hydroxylase (clone 3156.5, T2H), taxoid 7β-hydroxylase (T7H), taxoid 5α-hydroxylase (T5OH), taxoid 10β-hydroxylase (T10H), and taxoid 13α-hydroxylase (T13OH). Black boxes indicate identical residues; grey boxes indicate identical residues for at least three of the sequences. The substitutions (S56P, Q159K, I196M, N215K, A222P, L254Q, S259R, L263V, S278A, F279Y, K280E, V313M, and T440K) observed between the clone 3156 sequence variants (asterisks) and the predicted second substrate recognition site (dashed line) are indicated above the T2OH sequence.

CHAPTER FOUR

Comparative specificity and kinetic analyses of taxoid-O-acetyltransferases. Heterologous expression and characterization of a second taxadien-5α-ol-O-acetyltransferase

Abstract

In addition to the anticancer drug Taxol, yew (Taxus) species produce a large variety of other taxane diterpenoids which differ mainly in the type of acyl and aroyl groups appended to the many hydroxyl functions on the taxane core; acetate esters are particularly common. Taxol bears an acetate at C10 and another at C4, the latter thought to originate by intramolecular migration of a C5 acetate function in the process of oxetane ring formation, but many other naturally occurring taxoids bear acetate groups at C1, C2, C7, C9, and C13, in addition to C5 and C10. cDNAs encoding a taxoid 5α-O-acetyltransferase (taxadien-5α-ol as substrate) and a taxoid 10β-O-acetyltransferase (10-deacetylbaccatin III as substrate) have been isolated from a recently isolated family of Taxus acyl/aroyltransferase clones. To explore the origins of other acetylated taxoids, the group of recombinant Taxus acyltransferases was investigated with a range of polyhydroxylated taxoids as substrates. From this survey, a new acetyltransferase clone (denoted TAX19) was identified that encoded an enzyme capable of acetylating taxadien-5α-ol with activity comparable to that of the previously identified 5α-O-acetyltransferase (clone TAX1). However, when these two recombinant enzymes were presented with taxadien-triol and tetraol substrates, they exhibited different regiospecificities. The TAX1 enzyme preferentially acetylates the "northern" hemisphere hydroxyls at C9 and C10, whereas the TAX19 enzyme preferentially acetates the "east-west" pole positions at C5 and C13. The TAX1 enzyme possesses the lowest K_M value with taxadien-5α-ol (an early pathway metabolite) as substrate, with much higher K_M values for the polyhydroxylated taxoid substrates, whereas the TAX19 enzyme possesses lower K_M values (than the TAX1 transferase) for all taxoid substrates tested. These results suggest that the TAX1 acyltransferase may function at an early step of taxoid

metabolism, and that the TAX19 acyltransferase, because of its broader specificity for polyhydroxylated taxoids, may function later in metabolism and be responsible for the production of numerous acetylated taxoids.

Introduction

Taxoids (taxane diterpenoids), isolated from yew (Taxus) species, consist of a group of highly functionalized diterpenes, most of which possess the unique pentamethyl [9.3.1.0]^{3,8} tricyclopentadecane (taxane) skeleton like that found on the anticancer drug Taxol [1]. Although Taxol and its precursors for semisynthesis are arguably the most important taxoids, on examination of a compendium of the naturally occurring taxoids [2] reveals a large assortment of other compounds possessing positionally different acylation patterns and a variety of acyl and aroyl substitutions. These latter functional groups include acetyl, propionoyl and butyroyl, and their hydroxylated and methylated derivatives, tigloyl and benzoyl esters, and amino acid derivatives, such as cinnamoyl, aminophenylpropanoyl (Winterstein's acid), and phenylisoserinoyl esters, as well as occasional glycosyl groups. Taxol (Fig. 1, generic name paclitaxel) bears acetate groups at C4 and C10, a benzoate at C2, and the unusual N-benzoylphenylisoserine side chain appended at C13 (The C2-benzoate, C13-side chain, and C4-acetate are important pharmacophores in promoting tubulin binding that underlies the cytotoxic mode of action of Taxol [3]). However, taxoids bearing acetate groups variously at C1, C2, C5, C7, C9, C10, and C13 positions are far more abundant than Taxol and its congeners.

The rationale for the production of such a vast assortment of acetylated taxoids is unknown. Some of these metabolites may be relevant intermediates, or may simply represent the consequence of promiscuous acyltransferase activity, while others may play a role in plant defense in possessing antifeedant [4] and antifungal [5, 6] activities, and toxicity towards mammals [7, 8]. What is clear is that Taxus, both intact plants and derived cell cultures [9], direct considerable pathway flux to the production of acetylated taxoids other than Taxol, and

that any approach to improving the production yields of Taxol and its immediate precursors must take into account these apparently diversionary taxoid biosynthetic side-routes.

Considerable progress has been made in elucidating the biosynthesis of Taxol and other taxoids in yew [10, 11]. These efforts have led to the isolation of many genes encoding enzymes of the pathways, including genes for taxadiene synthase, the committed step [12], several cytochrome P450 taxoid hydroxlases [13-17] and all of the acyl and aroyltransferases thought to be involved in Taxol production. The latter are represented by the C13-side chain transferase [18] and N-benzoyltransferase [19], the taxoid C2-O-benzoyltransferase [20] and C10-O-acetyltranferase [21], and the C5-O-acetyltransferase that operates on taxa-4(20),11(12)-dien-5α-ol as a presumptive early pathway step [22]. The C4-acetate function of Taxol (Fig. 1) is thought to arise by intramolecular migration of the C5-acetate group in the process of oxetane (D-ring) formation [23] (Fig. 2).

Genes encoding the acyl and aroyltransferases of Taxol biosynthesis have been cloned by several strategies employing cDNA libraries derived from transcripts isolated from Taxus cell cultures induced with methyl jasmonate for increased Taxol production [11]. These approaches, in addition to the random sequencing of an EST library from similarly induced Taxus cells [24], has now yielded a family of 16 closely related acyltransferase clones, including the above described five which have been characterized by heterologous functional expression in Escherichia coli. The recent acquisition of this family of presumptive taxoid acyl/aroyltransferase clones has provided the opportunity to broaden our search for genes relevant to taxoid metabolism, with immediate focus on those responsible for the origin of acetylated taxoids and with a long term view to manipulate these genes so as to suppress side-routes and promote pathway flux towards Taxol. In this paper, we report the cloning and

characterization of a new taxoid acetyltransferase (denoted TAX19), and a comparison of the properties of the recombinant enzyme with those of previously isolated taxoid-O-acetyltransferases (TAX1 and TAX6), and we discuss the implications of the finding for the biosynthesis of Taxol and related acetylated taxoids.

Materials and Methods

Substrates

The preparation of racemic taxadien-5α-ol, (+)-taxadien-5α-acetoxy-10β-ol, (+)-taxadien-5α-acetoxy-2α,10β-diol, (+)-taxadien-5α,9α,10β,13α-tetraol, and (+)-taxadien-5α-acetoxy-9α,10β,13α-triol [16, 25-27] have been described. (+)-Taxadien-5α,10β-diol was generated by deacetylation of the 5α-monoacetate [26] using magnesium methoxide [16]. Authentic 10-deacetylbaccatin III was kindly provided by David Bailey of Hauser Chemical Research (Boulder, CO), and (+)-taxadien-2α,5α,10β-triol and (+)-taxadien-2α,5α-diol were generously provided by Tohru Horiguchi and Robert Williams of Colorado State University (Fort Collins, CO). (+)-Taxadien-5α,13α-diol and 2α-hydroxytaxusin were biosynthetically derived from the taxoid 13α-hydroxylase and the taxoid 2α-hydroxylase, respectively, using the corresponding taxadien-5α-ol and taxusin substrates [14, 17]. Acetyl CoA as the sodium salt was purchased from Sigma, and [^{3}H]acetyl CoA (2.83 Ci/mmol) was purchased from New England Nuclear and was diluted with unlabeled substrate to 45 Ci/mol at a working concentration of 1 mM.

Heterologous expression in E. coli and functional screening

Data mining of a Taxus EST library [24] yielded seven new full-length acyltransferase clones which were subcloned from their original pBluescript vector [24] into pSBET [28], and then individually transformed into BL21(DE3) and BL21 star (DE3) cells (Invitrogen) for expression as previously described [19]. A total of 16 acyltransferase clones, originating from both the EST library [24] and a previous homology-based screen of an induced Taxus cell cDNA library [22], were tested for function of the corresponding expressed enzyme with available taxoid substrates, including taxadien-5α-ol, taxadien-2α,5α-diol, taxadien-5α,13α-diol, taxadien-5α,10β-diol, taxadien-2α,5α,10β-triol, taxadien-5α,9α,10β,13α-tetraol, taxadien-5α-acetoxy-9α,10β,13α-triol (Table 1), and 10-deacetylbaccatin III (Fig. 1). Thus, soluble enzyme preparations from transformed E. coli cultures expressing each acyltransferase were prepared as previously described [19]. Each taxoid substrate (100 μM) was added to a 10 mL screw-capped vial, along with 50 μL (~500 μg protein) of the soluble enzyme extract, [^{3}H]acetyl CoA (100 μM, 45 Ci/mol), 50 mM Mopso buffer (pH 7.5) to a final volume of 200 μL, and the mixture was then shaken for 16 h at 31°C. The reaction products and residual taxoid substrate were then isolated by partitioning between 1 mL of brine and ether (2 x 2 mL). The pooled ether extracts were dried, and the resulting residue was redissolved in CH_3CN and analyzed by radio-HPLC with a method previously described [16].

Partial purification of recombinant acetyltransferases

Following the above screening, and prior to purification, the soluble enzyme extract of E. coli expressing the target clone was analyzed by SDS-PAGE and Coomassie brilliant blue staining [29] to verify production of the recombinant protein (~50 KDa) relative to comparable

preparation from the negative control harboring empty vector. E. coli cultures confirmed to express the target acyltransferase gene were grown in 1 L LB media (E. coli harboring TAX1 was grown in the presence of 1 M sorbitol and 2.5 mM betaine to reduce the formation of inclusion bodies [30]), shaken at 37°C until cell density increased to A_{600} ~0.5, and then induced with IPTG (0.5 mM) and grown for another 24 h at 18°C. The transformed bacteria were harvested by centrifugation (6000 x g for 20 min), washed with phosphate buffered saline, and centrifuged again. The pelleted cells were resuspended in extraction buffer (50 mM Mopso, pH 7.2, with 5% v/v glycerol, and 0.5 mM DTT), lysed by sonication (3 x 30 s) on ice using a Virsonic 475 (Virtis, Gardiner, NY) at medium power with the 0.5 inch probe. The derived homogenate was centrifuged (10,000 x g) for 20 min, and the resulting supernatant was centrifuged again at 45,000 x g for 1 h to provide the soluble enzyme extract. Using a Bio-Rad BioLogic LP pump with UV-monitoring at 254 nm, the protein extract (50 mL) was loaded on to a diethylaminoethyl-cellulose (10 g, Whatman DE-52, Clifton, NJ) column that was previously equilibrated with 50 mM Mopso, pH 7.2, containing 5% v/v glycerol, 5 mM $MgCl_2$, and 1 mM DTT (equilibration buffer) and eluted with a linear NaCl gradient (0-100 mM; 200 mL, 2 ml/min) in the same buffer. Fractions containing the target enzyme (eluting between 30-65 mM NaCl), as determined by the above assay, were pooled and loaded (2 mL/min) onto a ceramic hydroxyapatite (10 g, BioRad, Hercules, CA) column preequilibrated with the above equilibration buffer. The column was washed (3 mL/min) with 25 mL of equilibration buffer, followed by 50 mL of 50 mM Mopso, pH 7.2, containing 1 M NaCl, then again with 25 mL of equilibration buffer, followed by elution with a linear gradient of 0 to 50 mM potassium phosphate buffer (pH 7.2, 200 mL, 3 mL/min). Fractions containing the acetyltransferase (eluting between 10-25 mM), as determined by assay, were analyzed by SDS-PAGE with

Coomassie brilliant blue staining, and these fractions were pooled, concentrated using Amicon Centriprep YM-30 centrifugal concentrator, and used for subsequent characterization studies. An aliquot (12 μg) of the pooled concentrated protein was analyzed by SDS-PAGE to determine the relative percentage of recombinant protein with respect to total protein present. One liter of E. coli harboring TAX19 yielded ~5 mg of partially purified acyltransferase (60% pure); one liter of E. coli harboring TAX1 culture yielded ~1 mg of partially purified acyltransferase (40% pure). TAX1 preparations were scaled-up five-fold for routine use.

Product identification and enzyme characterization

After establishing reaction linearity with respect to protein concentration and time, the pH optimum of the purified recombinant acetyltransferase was determined in Tris (pH 6.5-8.0), Bistris-propane (pH 6.5-9.0) and phosphate (pH 5.0-9.5) buffers (each at 100 mM) over intervals of 0.5 pH units. Assays were performed for 1 min at 31°C with 100 μM of taxadien-tetraol as co-substrate, 15 μg of partially purified enzyme, the appropriate buffer, and 100 μM [^{3}H]acetyl CoA (45 Ci/mol) in a total assay volume of 200 μL. Reaction products were extracted to partition between 1 mL brine and ether (2 x 2 mL), the pooled ether extracts were dried, the residue was redissolved in acetone, and an aliquot was counted by scintillation spectrometry from which rate values were determined based on the specific activity of [^{3}H]acetyl CoA.

K_M values (assays in 100 mM phosphate, pH 8.0) for taxadien-5α-ol, taxadien-2α,5α-diol, and taxadien-2α,5α,10β-triol (all at concentration range of 2-100 μM), taxadien-5α,9α,10β,13α-tetraol (at concentration range 5-300 μM), and taxadien-5α-acetoxy-9α,10β,13α-triol (at concentration range 5-500 μM) were determined by Eadie-Hofstee plotting

using Microsoft Excel software. The reported values are the means ± SD of two independent replicates.

For structural elucidation of biosynthetic products, the assays were scaled-up sufficiently (in 100 mM phosphate buffer, pH 8.0, with 5 mM DTT and 100 μM of each acetyl CoA and taxoid substrate; 4 h at 31°C) to generate about 1 mg of material. The products were extracted as before and purified by HPLC on a Metachem 5μ Taxsil column with an isocratic gradient (2:3 CH_3CN:H_2O). The purified material was concentrated to remove CH_3CN, NaCl was added to the residual aqueous phase, and the product was extracted twice with an equal volume diethyl ether. The ether was evaporated, and the residual material was lyophilized and then dissolved in 100% $CDCl_3$ for NMR analysis. ^{1}H-NMR, NOESY, COSY, and TOCSY experiments were performed on a Varian Mercury 300 MHz instrument. Coupled GC-MS analyses were performed on a Hewlett-Packard 6890 MSD system (electron impact at 70 eV ionizing voltage) using a Phenomenex ZB-5 column (30 m X 0.25 mm, 5% phenyl polysiloxane) and cool on-column injection, with programming from 40°C at 30°C/min to 220°C, then 2°C/min to 260°C, and finally 20°C/min to 320°C.

Results and Discussion

Product identification and regiospecificity analyses

Screening of the acyl/aroyltransferase clones verified to express the recombinant enzyme by SDS-PAGE, was conducted in soluble protein extracts of the corresponding transformed bacteria with all available taxoid substrates and acetyl CoA as co-substrate, and with product assessment by GC-MS and radio-HPLC. The previously described recombinant 10-

deacetylbaccatin III-10-O-acetyltransferase (TAX6) [21], was shown to acetylate taxadien-5α,9α,10β,13α-tetraol to the corresponding tetraol-9-acetate, tetraol-10-acetate, and tetraol-9,10-diacetate, and to convert taxadien-5α-acetoxy-9α,10β,13α-triol to the corresponding tetraol-5,9-diacetate and tetraol-5,10-diacetate. None of the other test substrates was measurable functional with this enzyme. These newly described activities of the deacetylbaccatin III-acetyltransferase (see Fig. 1) are consistent with the utilization of moderately to highly functionalized taxoids by this enzyme, with a common "northern hemisphere" regioselectivity. The previously characterized taxoid sidechain-N-benzoyltransferase (TAX10) [19], and a new EST acyltransferase clone (designated TAX14), were found to selectively convert taxadien-5α,13α-diol [14] to the same diol monoacetate, whereas the previously described taxoid 5α-acetyltransferase (TAX1) [22] and a previously undefined acyltransferase (TAX19) were both found to acetylate a range of taxoid substrates, including taxadien-5α-ol, taxadien-5α,10β-diol, taxadien-5α,13α-diol, taxadien-2α,5α,10β-triol, taxadien-5α,9α,10β,13α-tetraol, and taxadien-5α-acetoxy-9α,10β,13α-triol. The TAX19 enzyme was also able to utilize taxadien-2α,5α-diol as substrate (Table 1). Because of the range of substrates utilized and sequences considerations (see below), the TAX1 and TAX19 acetyltransferases were examined in greater detail.

Substrate selectivity and regioselectivity of TAX1 and TAX19

Both TAX1 and TAX19 acyltransferases were able to convert taxadien-5α-ol to taxadien-5α-yl acetate (Table 1); this is the original activity for which TAX1 was designated a taxoid 5α-O-acetyltransferase [22]. Like the TAX10 and TAX14 acyltransferases described above, the TAX1 and TAX19 enzymes were also capable of acetylating taxadien-5α,13α-diol to the same diol monoacetate, and the latter two could additionally acetylate taxadien-5α,10β-diol to

taxadien-5α-acetoxy-10β-ol (product identification by HPLC-based comparison to the authentic standard [13]). One interesting difference with respect to substrate utilization was demonstrated by the ability of the TAX19 acetyltransferase to acetylate taxadien-2α,5α-diol; the TAX1 enzyme had no detectable activity with this substrate. The acetylated product of taxadien-2α,5α-diol was analyzed by GC-MS ($\underline{R_t}$ 17.61 min) and yielded a fragmentation pattern consistent with a diol monoacetate. The parent ion (P^+) was observed at $\underline{m/z}$ 346 in addition to diagnostic fragment ions at $\underline{m/z}$ 328 (P^+-H_2O), 313 (P^+-H_2O-CH_3), 286 (P^+-CH_3COOH), 268 (P^+-CH_3COOH-H_2O), and 253 (P^+-CH_3COOH- H_2O-CH_3). Subsequent studies with taxadien-2α,5α,10β-triol as substrate revealed the first example of a regiospecific difference between the TAX1 and TAX19 acetyltransferases. The TAX19 enzyme was capable of acetylating this taxoid triol to taxadien-5α-acetoxy-2α,10β-diol (identification by GC-MS comparison to the authentic standard [27]). The regiospecific acetylation at the C5 hydroxyl (but not the C2 hydroxyl) of this 2,5,10-triol suggests that the TAX19 transferase also regiospecifically acetylated the C5 position of the above taxadien-2,5-diol (but this identification was not confirmed). TAX1 transferase, on the other hand, also acetylated this taxadien-triol but the product had a different HPLC retention than did the C5-acetoxy derivative produced by TAX19, indicating that acetylation by TAX1 occurred at either the C2 or C10 positions (likely the C10-hydroxyl; see below).

The TAX1 and TAX19 transferases were also both capable of converting taxadien-5α,9α,10β,13α-tetraol to a number of different acetylated products (Table 2). One product formed by the TAX1 enzyme (25% of product mix; $\underline{R_t}$ 27 min by HPLC) was purified by HPLC (~1 mg, >95% purity) and the structure determined by ^{1}H-NMR: δ: 0.91 (s, CH_3), 1.01 (s, CH_3), 1.2 (dd, H14α, $\underline{J}$ = 4.2 Hz), 1.43 (s, CH_3), 1.53 (m, H7), 1.66 (m, H2), 1.7 (m, H1), 1.74 (m, H6),

2.02 (s, $COCH_3$), 2.16 (d, allylic-CH_3, J = 1.2 Hz), 2.87 (dt, H14β, J = 3.6, 9.3, 15.6 Hz), 3.00 (br s, H3), 4.35 (t, H5, J = 3 Hz), 4.43 (br d, H13, J = 7.2 Hz), 4.72 (d, H20, J = 1.2 Hz), 4.90 (d, H10, J = 11.7 Hz), 5.11 (s, H20), 5.60 (d, H9, J = 12 Hz). Based on these proton assignments, and spectrometric comparison with the substrate tetraol and its peracetylated derivatives (taxusin), this product was confirmed as taxadien-9α-acetoxy-5α,10β,13α-triol. A second product (55% of the product mix) formed by the TAX1 transferase with taxadien-tetraol substrate had a slightly later retention time than the first (R_t 28 min by HPLC), and this product was also purified (~1 mg, >90% purity) and the structure determined by ^{1}H-NMR: δ: 0.89 (s, CH_3), 0.91 (s, CH_3), 1.24 (dd, H14α, J = 4.5 Hz), 1.40 (s, CH_3), 1.68 (m, H2), 1.7 (m, H6), 1.7 (m, H1), 1.8 (m, H7), 2.11 (s, $COCH_3$), 2.22 (d, allylic-CH_3, J = 1.5 Hz), 2.81 (dt, H14β, J = 3.6, 8.9, 14.7 Hz), 3.25 (br s, H3), 4.12 (d, H9, J = 9.9 Hz), 4.34 (br s, H5), 4.37 (br s, H13), 4.71 (s, H20), 5.07 (s, H20), 5.89 (d, H10, J = 9.9 Hz). Based on these assignments, and comparison with reference NMR spectra of the starting tetraol, taxusin, and other tetraol derivatives [16], this product was confirmed as taxadien-10β-acetoxy-5α,9α,13α-triol. Essentially similar methods and comparison to authentic standards [16] were used to determine the identities of the remaining TAX1 and TAX19 enzyme products, and to demonstrate that the major product formed by the TAX19 acetyltransferase from taxadien-5α,9α,10β,13α-tetraol was taxadien-13α-acetoxy-5α,9α,10β-triol (Table 2). With the TAX1 transferase the major product formed from taxadien-5α-acetoxy-9α,10β,13α-triol from was taxadien-5α,9α,10β-triacetoxy-13α-ol, and with the TAX19 transferase, the major product from this substrate was taxadien-5α,13α-diacetoxy-9α,10β-diol (Table 2). These results are entirely consistent with the regioselectivities of the respective enzymes observed with the taxadien-tetraol substrate.

The TAX19 transferase acetylated taxadien-2α,5α,10β-triol principally to taxadien-5α-acetoxy-2α,10β-diol (by comparison to the authentic standard [27]), whereas the major product of the TAX1 enzyme with this triol substrate had a slightly longer retention time by GC (19.87 min) and a mass spectrum entirely characteristic of a triol monoacetate. The parent ion (P^+) was not observed at m/z 362; however, diagnostic fragment ions were observed at m/z 344 (P^+-H_2O), 329 (P^+-H_2O-CH_3), 302 (P^+-CH_3COOH), 284 (P^+-CH_3COOH-H_2O), 269 (P^+-CH_3COOH-H_2O-CH_3), 251 (P^+-CH_3COOH-H_2O-CH_3-H_2O). The triol monoacetate produced by TAX1 was not in sufficient quantity to permit NMR analysis; however, the regiospecificity of this acetylation could be inferred from other results. Because TAX1 does not acetylate taxadien-2α,5α,10β-triol at C5, the only other possible positions are either the C2 or C10 hydroxyl. TAX1 does not acetylate taxadien-2α,5α-diol but is capable of acetylating taxadien-5α,10β-diol to the C5-monoacetate derivative, and is capable of acetylated taxadien-5α,9α,10β,13α-tetraol to the C9-acetate and the C10-acetate derivatives; note again that TAX19 preferentially acetylates the "east-west hemisphere" hydroxyl groups (C5 and C13 of this substrate). Because TAX1 does not acetylate the C2-hydroxyl of taxadien-2α,5α-diol (nor that of 2α-hydroxytaxusin [17]) but rather has a tendency to acetylate the C10-hydroxyl of polyhydroxylated taxoid substrates, it is very likely that the TAX1 transferase acetylates taxadien-2α,5α,10β-triol regioselectively at C10. Given the differences in selectivity observed, it is of note that both TAX1 and TAX19 transferases are capable of efficiently acetylating the C5-hydroxyl of taxadien-5α-ol and taxadien-5α,10β-diol. These observations suggest that the presence of a C2-hydroxyl and/or the presence of multiple hydroxyl groups in the substrate promote differential steric effects that expose regiochemical differences in the acetylation capability of these enzymes. Thus, the TAX1 and TAX19 transferases have apparently similar activities with less functionalized

taxoids; however, at the level of triol and tetraol substrates, and with 2α-hydroxy-containing diols, these enzymes demonstrate fairly precise differences in regioselectivity.

Kinetic analyses of TAX1 and TAX19

Limited substrate availability prevented kinetic assessment with the taxadien 5,13- and 5,10-diols but full kinetic assessment was possible with the remaining test substrates (Table 1). The TAX1 and TAX19 acetyltransferases exhibit the lowest K_M values for taxadienol, with respective k_{cat} values of 4.3 s^{-1} and 29.6 s^{-1} and kinetic efficiencies that differed by a factor of ten (Table 1). TAX19 utilized taxadien-2α,5α,10β-triol much more efficiently than did TAX1, again probably due to the influence of the C2-hydroxyl as noted above. Both enzymes utilized the taxadien-tetraol with roughly comparable efficiency. A noteable difference was observed with taxadien-5α-acetoxy-9α,10β,13α-triol as substrate, for which both enzymes exhibited substantially higher K_M and k_{cat} values. TAX1 also demonstrated an interesting trend, in that as the level of substrate functionality increased, both K_M and k_{cat} values increased; no trend of this type was observed with TAX19, which exhibited roughly comparable kinetics for taxadien-monool, diol, triol, and tetraol substrates.

Both TAX1 and TAX19 exhibited the lowest K_M values (and respectable turnover rates) for taxadien-5α-ol, an early intermediate of Taxol biosynthesis [31], suggesting that acetylation at C5 is of relevance in vivo as an early pathway step. Based on utilization of the other test substrates, TAX19 appears to be the more promiscuous of the two transferases but both enzymes do catalyze reactions "off pathway" for Taxol production (i.e., C9 acetylation by TAX1 and C13-acetylation by TAX19). Based on the high K_M values for both enzymes in the utilization of taxadien-5α-acetoxy-9α,10β,13α-triol, it might be argued that C5-acetoxy taxadien polyols are

not relevant substrates for these enzymes in vivo; however, the k_{cat} values for this substrate were also remarkable high, thus yielding quite respectable catalytic efficiencies (Table 1). The K_M values for acetyl CoA with TAX1 was previously reported as 5.5 μM [32]; the K_M value for acetyl CoA with TAX19 was determined in this study to be 5.2 ± 2.5 μM. Kinetic parameters in the present study were measured by total (multiple) product formation. It is appreciated that these kinetic values represent a mixture of reaction channels and (presently indistinguishable) binding mode for the taxoid substrate and/or acetyl CoA. The kinetic constants for TAX1 and TAX19 compare favorably with those of the 10-deacetylbaccatin III-10-O-acetyltransferase (TAX6) with K_M values of 10 μM and 8 μM for 10-deacetylbaccatin III and acetyl CoA, respectively [21].

Sequence analyses of acetyltransferases

The TAX19 cDNA (orf 1329 bp) encodes a 442-residue protein with a calculated molecular weight of 49,290. This size is consistent with the results of SDS-PAGE analysis of the recombinant protein and is very similar to that of the TAX1 taxadienol-5α-O-acetyltransferase previously described [22, 32]. The cDNA sequence does not appear to encode protein N-terminal targeting information, consistent with the presumptive cytosolic localization of the enzyme and its operationally soluble nature. Examination of the deduced amino acid sequence (Fig. 3) reveals the highly conserved HXXXDG catalytic triad (aa 164-169) found in other acyltransferases [11, 33-35]. Site-directed mutagenesis of the histidine motif has shown that it is essential for catalytic activity, and it has been suggested that this residue acts as a general base in the catalytic transfer of the acyl moiety from acyl CoA to the substrate hydroxyl group [33, 34]. It has also been reported that a cysteine residue is important for activity of O-

acetyltransferases [36]. An alignment (GCG program, Madison, WI, version 10 ; gap creation penalty of 3, gap extension penalty of 1) of the defined Taxus acyltranferases (Fig. 3), reveals two cysteines, in addition to that of the HXXXDG motif, that are highly conserved, one at position-96 (of TAX19), the other part of a CGG motif (aa 153-155). Another conserved element of this class of enzymes is the DFGWG motif (aa 375-379) [34].

Pair-wise comparisons (GeneDoc, version 2.6.002) of TAX1 and TAX19 reveals moderate sequence similarity (S) and identity (I) scores of 77% and 61%, respectively. Comparisons of the TAX1 andTAX19 sequences with the other described acyl/aroyltransferases of Taxol biosynthesis also showed similar scores (~60% I, ~75% S). However, one comparison did reveal very high homology; TAX14 and TAX19 exhibited identity and similarity scores of 87% and 93%, respectively. Although these sequences are highly homologous, these two enzymes only share one common substrate, taxadien-5α,13α-diol, whereas the TAX1 and TAX19 sequences are respectively less similar but utilize several common substrates with comparable activities; such differences are difficult to determine based on sequence alone. Neither of these acetyltransferases is highly abundant in the induced Taxus cell EST library [24] (TAX1 at 0.2‰ and TAX19 at 0.1‰) relative to the other acyltransferases of Taxol biosynthesis (all > 1.0‰). These low abundances may result from down regulation of the TAX1 and TAX19 genes upon methyl jasmonate elicitation of the Taxus cells; alterations in gene expression upon induction of Taxol biosynthesis are not yet well defined.

Conclusions

With the acyl and aroyltransferases involved in Taxol biosynthesis seemingly defined [11, 18, 19], we turned our attention to the origin of other acylated taxoids, particularly the numerous acetylated derivatives, and functionally screened all of the available Taxus acyltransferase clones with the newly acquired taxoid test substrates using acetyl CoA as co-substrate. From this family of taxoid acyltransferases, a new clone denoted TAX19 was found to possess taxadien-5α-O-acetyltransferase activity like that of the previously isolated TAX1 acetyltransferase [22]. Both TAX1 and TAX19 enzymes were subsequently shown to be capable of utilizing a range of polyhydroxylated taxoid substrates with differing regioselectivities, TAX1 with preference for C9 and C10 and TAX19 with preference with C13 and C5 acetylation. These two enzymes are capable of acetylating taxadien-5α-ol with respectable kinetics, suggesting a role in vivo.

Acetylation at C5 (i.e., following the first hydroxylation of the taxane core to taxadien-5α-ol [31]) would appear to be an early step of taxoid biosynthesis, the C5-acetylated product leading to subsequent hydroxylation at C10 [13] and establishing a structural element at C5 that is seemingly necessary for the eventual construction of the oxetane D-ring [23] (See Fig. 2). Although taxadien-5α-yl acetate has been demonstrated as a metabolite of Taxus [32, 37], the enzymology of the acetylation of taxadien-5α-ol established [32] and the corresponding gene isolated [22], it has not been possible to adequately evaluate the legitimacy of taxadien-5α-yl acetate as an intermediate in taxoid biosynthesis by feeding studies with Taxus cells because the uptake of this metabolite is very inefficient.

Of the two acyltransferases, TAX19 possesses the broader specificity for the taxoid substrates tested, and in general more favorable kinetics. Both enzymes catalyze reactions that could divert from the Taxol pathway by acetylation at C9 (TAX1) or C13 (TAX19). Nevertheless, the seemingly greater promiscuity of the TAX19 acyltransferase makes it the more likely enzyme involved in the production of abundant "off-pathway" acetylated taxoids and, thus, the better candidate for suppression of side-routes to increase pathway flux towards Taxol.

It is appreciated that the functional implications for TAX1 and TAX19 (and other acyltransferase TAX clones) derive from studies with a limited set of test substrates. At present, there are exceedingly few accessible test substrates with functional complexity between that of taxadien-tetraol and baccatin III (which was not a substrate for either TAX1 or TAX19 enzyme). It is also important to note that the functioning of acyltransferases in vivo is highly dependent on the type and concentration of acyl CoA and aroyl CoA co-substrates available, an issue not addressed here, and that, with sufficient time in vivo (as opposed to rapid in vitro assay) even kinetically favored acylations can contribute to the accumulation of dead-end metabolites. Given these limitations, it is nevertheless important to define, to the extent possible, the functions of the taxoid acyltransferases, because these enzymes catalyze critical steps of Taxol biosynthesis and are largely responsible for the diverse assortment of other taxoids found in yew species that, in the context of Taxol production, represent lost yield. The taxoid substrate and acyl CoA specificities, and the regioselectivities of at least nine more Taxus acyltransferases remain to be defined.

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Footnotes

1 Abbreviations used: BisTris-propane, 1,3-bis[tris(hydroxylmethyl)methylamino] propane; COSY, correlated spectroscopy; DTT, dithiothreitol; EST, expressed sequence tag; GCG, Genetics Computer Group; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; LB, Luria-Bertani media; MOPSO, 3-morpholino-2-hydroxypropane-sulfonic acid; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TOCSY, two-dimensional homonuclear total correlation spectroscopy; Tris, tris-(hydroxymethyl)-aminomethane.

Taxoid Substrate	TAX1	TAX19
Taxadien-5α-ol	K_M: 8.6 ± 0.5 μM k_{cat}: 4.28 s^{-1} k_{cat}/K_M: 0.50 μM^{-1} s^{-1} Taxadien-5α-yl acetate	K_M: 5.0 ± 0.3 μM k_{cat}: 29.59 s^{-1} k_{cat}/K_M: 5.92 μM^{-1} s^{-1} Taxadien-5α-yl acetate
Taxadien-2α,5α-diol	No Detectable Activity	K_M: 23.9 ± 5.6 μM k_{cat}: 52.13 s^{-1} k_{cat}/K_M: 2.19 μM^{-1} s^{-1} Taxadien-5α-acetoxy-2α-ol
Taxadien-2α,5α,10β-triol	K_M: 41.5 ± 0.2 μM k_{cat}: 5.77 s^{-1} k_{cat}/K_M: 0.14 μM^{-1} s^{-1} Taxadien-10β-acetoxy-2α,5α-diol	K_M: 12.9 ± 1.0 μM k_{cat}: 27.92 s^{-1} k_{cat}/K_M: 2.17 μM^{-1} s^{-1} Taxadien-5α-acetoxy-2α,10β-diol
Taxadien-5α,9α,10β,13α-tetraol (taxusin tetraol)	K_M: 45.3 ± 3.0 μM k_{cat}: 11.91 s^{-1} k_{cat}/K_M: 0.26 μM^{-1} s^{-1} Taxadien-10β-acetoxy-5α,9α,13α-triol	K_M: 17.3 ± 0.2 μM k_{cat}: 15.47 s^{-1} k_{cat}/K_M: 0.90 μM^{-1} s^{-1} Taxadien-13α-acetoxy-5α,9α,10β-triol
Taxadien-5α-acetoxy-9α,10β,13α-triol	K_M: 279.4 ± 9.0 μM k_{cat}: 297.92 s^{-1} k_{cat}/K_M: 1.07 μM^{-1} s^{-1} Taxadien-5α,9α,10β-triacetoxy-13α-ol	K_M: 83.5 ± 8.3 μM k_{cat}: 144.97 s^{-1} k_{cat}/K_M: 1.74 μM^{-1} s^{-1} Taxadien-5α,13α-diacetoxy-9α,10β-diol

Table 1

Major product formed by, and kinetic analyses of, TAX1 and TAX19 acetyltransferases. The taxoid test substrates are shown in the left column, along with the corresponding K_M, k_{cat}, and catalytic efficiency values. For position numbering, see Figure 1.

TAX1				TAX19			
Tetraol		Tetraol-5OAc		Tetraol		Tetraol-5OAc	
				13	45%		
9	25%	9	15%	5	5%	9	15%
10	55%	10	35%	9	10%	10	20%
9,10	20%	9,10	50%	10	20%	13	65%
				5,13	20%		

Table 2

Distribution of products formed by TAX1 and TAX19 acetyltransferases with taxadien-5α,9α,10β,13α-tetraol (Tetraol) and taxadien-5α-acetoxy-9α,10β,13α-triol (Tetraol-5OAc) as substrates. The acetylated hydroxyl group(s) and the relative percentage conversion rates are shown for each substrate.

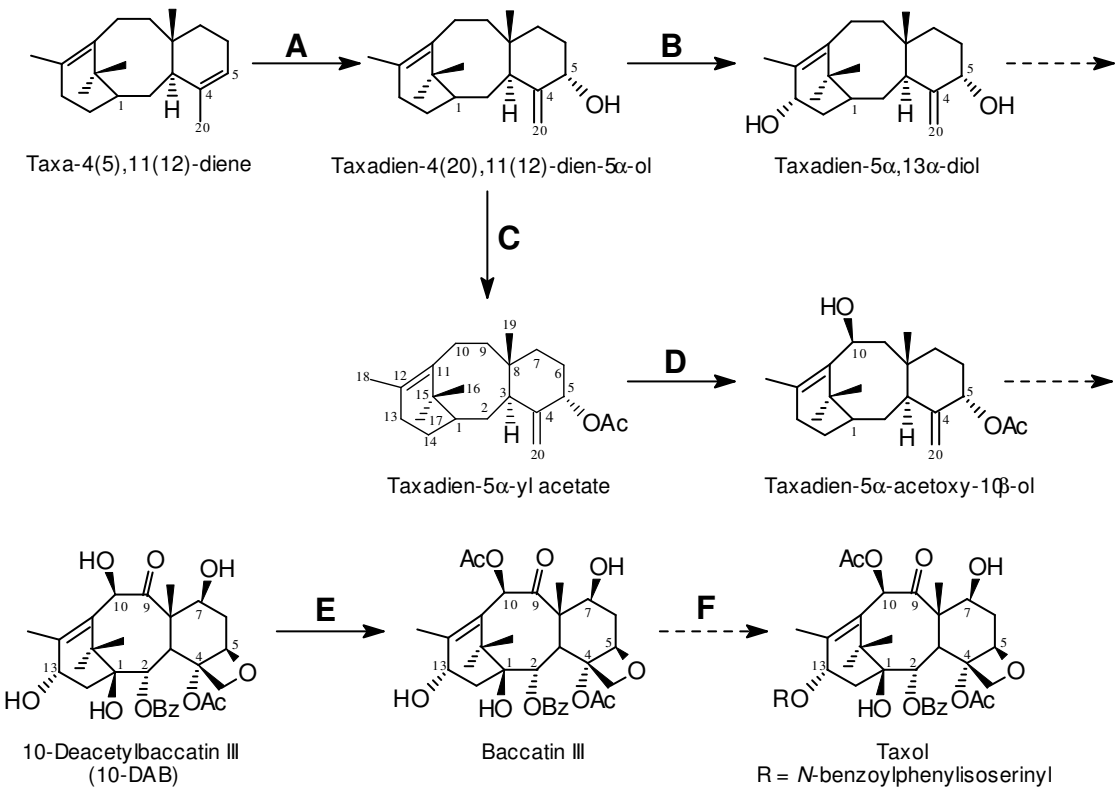
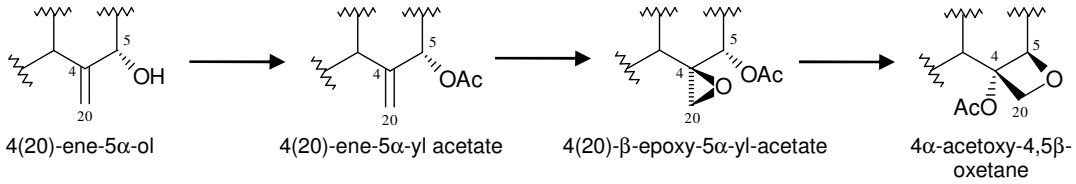


Figure 1. Outline of the Taxol biosynthetic pathway. Hydroxylation of the committed precursor taxa-4(5),11(12)-diene to taxadien-5α-ol by the taxoid 5α-hydroxylase (A), hydroxylation of taxadien-5α-ol by the taxoid 13α-hydroxylase (B), acetylation of taxadien-5α-ol by the taxadienol-O-acetyltransferase (TAX1 or TAX19) (C), hydroxylation of taxadien-5α-yl acetate by the taxoid 10β-hydroxylase (D), acetylation of 10-deacetylbaccatin III (10-DAB) by the 10-DAB-10-O-acetyltranferase (TAX6) (E), and the sidechain attachment to baccatin III to form Taxol (F) are depicted. Broken arrows indicate multiple steps, several of which remain undefined.



4(20)-ene-5α-ol | 4(20)-ene-5α-yl acetate | 4(20)-β-epoxy-5α-yl-acetate | 4α-acetoxy-4,5β-oxetane

Figure 2. A postulated biosynthetic scheme for oxetane ring formation in late-stage taxoids. The 4(20)-ene-5α-ol functional grouping is converted to the 4(20)-ene-5α-yl acetate, followed by epoxidation to the corresponding 4(20)-β-epoxy-5α-acetoxy derivative, and then intramolecular rearrangement to the 4α-acetoxy-4,5β-oxetane moiety.

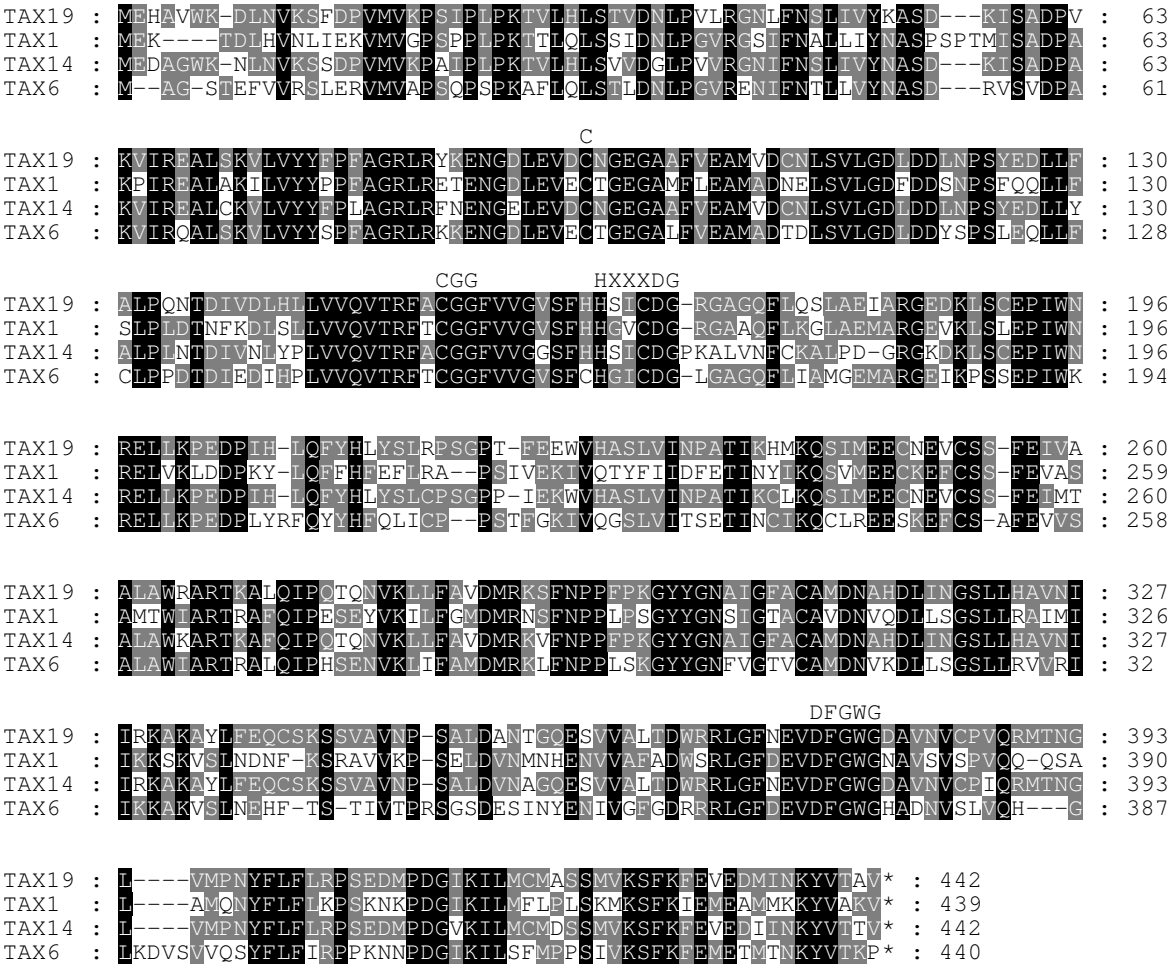


Figure 3. The deduced amino acid sequence alignment of taxoid acetyltransferases: TAX19 (taxoid 5α-acetyltransferase), TAX1 (previously identified taxoid 5α-acetyltransferase), TAX14 (acyltransferase of taxadien-5α,13α-diol), and TAX6 (10-DAB-10-O-acetyltranferase). Black boxes indicate identical residues; grey boxes indicate identical residues for at least two of the sequences. The conserved motifs are indicated.

CHAPTER FIVE

CONCLUSIONS

The isolation of genes encoding mid-pathway cytochrome P450 hydroxylases of the Taxol biosynthetic pathway has been more difficult to approach than those of early pathway steps because of the uncertainties about the exact sequence of these reactions and their intermediates, and because of the lack of readily available test substrates. For this reason, a more accessible 'surrogate' substrate, (+)-taxusin (taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol tetraacetate), was used to test the function of previously uncharacterized cytochrome P450 clones. This screen led to the identification of a taxoid 2α-hydroxylase and taxoid 7β-hydroxylase. The isolation of these newly described cytochrome P450 hydroxylase genes has now defined two of the nine oxygenation steps involved in the biosynthesis of Taxol. Manipulation of these genes could prove useful in the improved production of Taxol in yew and for the preparation of other 2α-hydroxy and 7β-hydroxy taxoids as starting materials for subsequent modification at these positions.

In addition to oxygenations, acylations constitute prominent steps of Taxol biosynthesis. An understanding of these reactions, and those responsible for side-route diversions of the pathway, is also important in improving Taxol yields. The cloning and characterization of a second taxadienol-5α-O-acetyltransferase is described, and a comparison with the previously-isolated 5α-O-acetyltransferase is reported. This new taxoid 5α-acetyltransferase is active in acetylating several positions of polyhydroxylated taxoid substrates, an observation which suggests the involvement of this enzyme in the formation of side-route taxoids that divert Taxol formation. Silencing of this gene (i.e., by the antisense RNA approach) could limit the formation

of side-route acetylated taxoids, to increase the flux of relevant intermediates to Taxol to give higher yields.

Although progress has been made in identifying the enzymes and genes involved in Taxol formation, for example in the successful acquisition of the taxoid 2α-hydroxylase [1] and taxoid 7β-hydroxylase [2] described here, several biosynthetic pathway steps still remain to be elucidated. Thus, cDNA clones encoding the presumptive cytochrome P450 C1β-, C2′-, and C9α-hydroxylases, and the putative C4,C20-epoxidase have yet to be identified. Uncertainties about the timing of these reactions and the relevant intermediates have limited synthetic efforts directed to the preparation of "surrogate" substrates for in vitro and in vivo studies and for test of function of the expressed genes. Nevertheless, the successful identification of the 2α- and 7β-hydroxylase has demonstrated the validity of this general approach.

The oxidation of the taxane C9-hydroxyl function to the corresponding ketone could also be mediated by a cytochrome P450 hydroxylase (via the ketone hydrate). This step is thought to occur relatively late in the pathway where uncertainties about the preceding steps, such as the timing of the oxetane ring formation, also constrain substrate design for test of function of the remaining cytochrome P450 candidate clones. On the other hand, the oxidation step may be catalyzed by a more typical pyridine nucleotide-dependant dehydrogenase. However, biochemical studies to define the type of enzyme involved in C9-oxidation must precede a cloning effort. A most interesting enzyme of the Taxol pathway is that presumed to catalyze the ring expansion of the epoxide to the oxetane function with migration of the 5α-acetoxy group. Little is known about this novel reaction, with regards to the enzyme type, the timing on the pathway, and the relevant substrate requirements. Again biochemical studies are required to

define the substrate(s) and product(s) of this unusual reaction before any attempt acquire the responsible gene.

As regards side chain assembly, two genes are still missing, that encoding the β-phenylalanine CoA ligase required for side chain activation as a prelude to transfer to baccatin III, and that encoding the presumed cytochrome P450-mediated C2′-side chain hydroxylase for the conversion of β-phenylalanoyl-baccatin III to phenylisoserinoyl-baccatin III as the penultimate step of Taxol biosynthesis. Given the great variety of different acyl and aroyl substitutions found among 350 naturally occurring taxoids, there must exist many other CoA ligases and acyl/aroyl transferases responsible for these largely diversionary modifications to the taxane core and C13 side chain. Understanding the origins of 'side-route' taxoids may be as important as elucidating the Taxol pathway itself for improving drug yields. Functional screening of candidate ligases, transferases, and cytochrome P450 oxygenases is in progress to define additional steps of taxoid metabolism.

Using induced Taxol production in Taxus cell cultures as a model system for understanding taxoid biosynthesis, enzymology and molecular genetics has been quite successful [3, 4]. However, biochemical and genetic gaps still exist in defining several pathway steps and in sequencing this extended series of reactions leading to Taxol. Efforts to fully elucidate this complex pathway, its side-routes and metabolite dead ends are underway, which will ultimately provide the insight about Taxol biosynthesis necessary for improving the production yields of this drug. It is also anticipated that new information about the organization, regulation, and evolutionary origins of this complex natural product pathway will be forthcoming. Eventually, it is expected that genetic engineering of Taxus cell cultures to improve rate-limiting steps and

suppress irrelevant taxoid biosynthetic pathways will lead to the development of a practical platform for the production of Taxol and related useful taxoids.

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