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Review

Taxol: A complex diterpenoid natural product with an evolutionarily obscure origin

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Taxol, a diterpenoid natural product first isolated from *Taxus brevifolia*, is one of today's better known anticancer drugs. Despite its clinical efficacy, the difficulty of establishing a secure and cost-effective supply of taxol has limited its use. However, its unique mode of action and efficacy against multiple forms of cancer has ensured continual efforts to achieve total and semisynthesis, as well as biotechnological production methods. Total synthesis is now possible but inefficient, so the production of taxol and related taxoids remains completely dependent on biomass derived from *Taxus sp*, with cell suspensions and collected plant materials as sources. The key to improving the supply of taxol and other clinically useful taxoids is the detailed elucidation of the taxoid biosynthesis pathway, which has been the subject of intense research. Many genes and enzymes in the *Taxus* pathway for taxoid biosynthesis have now been identified, although gaps remain. In addition to *Taxus sp*, taxoids are also synthesized by various endophytic fungi, which often live in association with *Taxus* trees, thus raising questions about the evolutionary origin of this complex diterpenoid pathway. In the future, it may be possible to improve taxoid synthesis through the genetic modification of *Taxus* cell cultures, by culturing endophytic fungi or by transferring the entire pathway into a heterologous expression host, such as *Saccharomyces cerevisiae*.

Key words: Taxol, paclitaxel, *Taxus*, *Endophytic fungi*, isoprenoids.

TAXOL – HISTORY, CLINICAL IMPACT AND PRODUCTION

During the 1950s, a joint scientific undertaking between the National Cancer Institute (NCI) and the United States Department of Agriculture (USDA) was set up to screen natural products and identify potent new anticancer drugs. This program led to the identification of taxol and camptothecin, which are still widely used today (Suffness and Wall, 1995). Taxol (generic name paclitaxel) (Figure 1) is probably the better known of the two and is the focus of this review. The compound was first isolated from the bark of the pacific yew tree *T. brevifolia*, and its complex, highly oxygenated diterpenoid structure was determined in 1971 (Wani et al., 1971). Taxol has become one of the most successful treatments for a variety of cancers (including ovarian, breast, lung, head and neck carcinomas and the AIDS-related Karposi's carcinoma) despite difficulties in generating a reliable supply (Goldspiel, 1997; Cragg et al., 1993). The genus *Taxus* is distributed throughout Asia, North and Central America and Europe, and all species and subspecies produce taxol-like compounds referred to as taxoids (Kingston et al., 2002). More than 350 structurally distinct taxoid compounds have been isolated (Baloglu and Kingston, 1999; Itokawa 2003). Furthermore, the search for additional sources and organisms capable of clinically useful biotransformations has shown that certain endophytic fungi isolated from *Taxus* trees can produce taxol and other taxoids.

Soon after its isolation, taxol was shown to have a unique mode of action based on shifting microtubule equilibrium towards assembly, resulting in abnormally sta-

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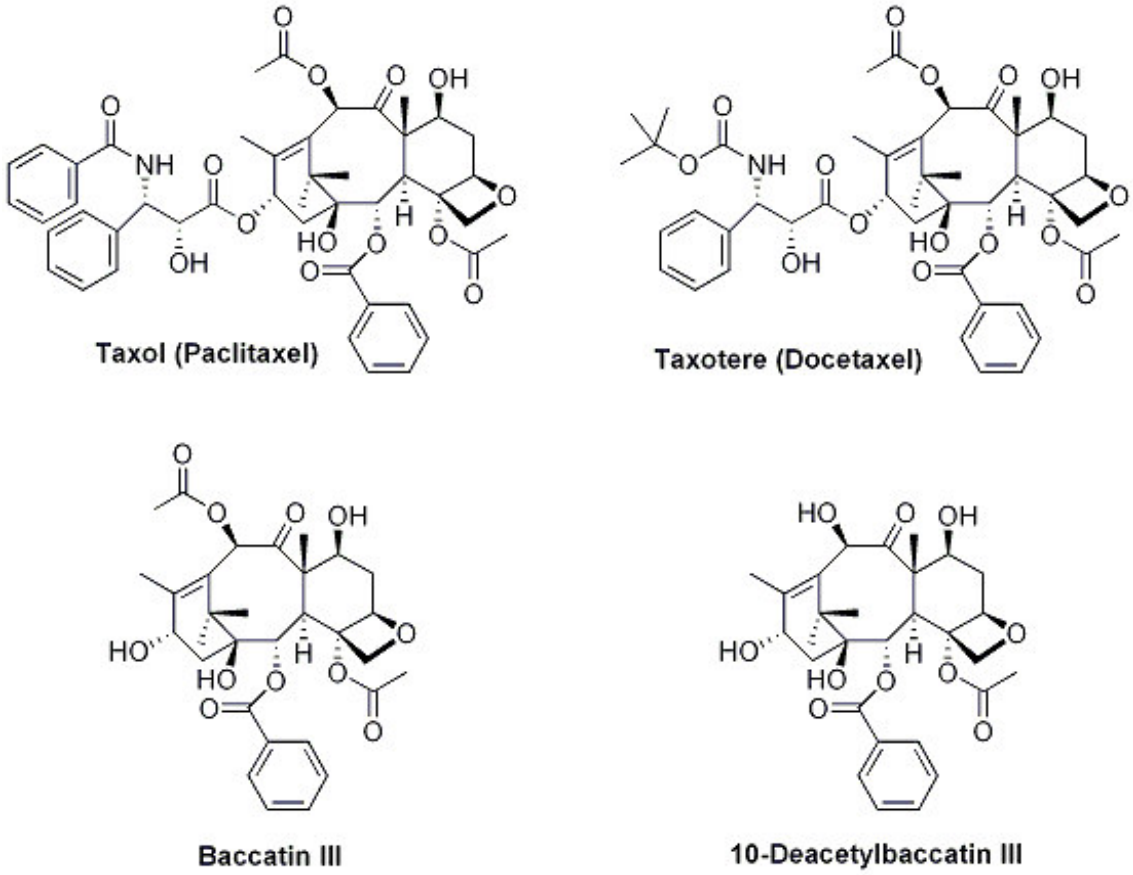

Figure 1. Structures of taxol, the semisynthetic derivative taxotere and the two taxol-precursors baccatin III and 10-deacetylbaccatin III.

ble microtubules that block the cell cycle in the G_2/M phase (Schiff et al., 1979). Even now, 40 years later, only a few compounds with the same mode of action are recognized, e.g. epothilones (Goodin, 2008). Taxol underwent clinical trials in the 1980s and was approved by the FDA for the treatment of refractory ovarian cancer in 1992 (Suffness and Wall, 1995). This was followed by approval for treatment of several other cancers (Oberlies and Kroll, 2004).

Today, taxol and its chemical derivative taxotere (Figure 1) are among the most widely used anticancer drugs, but they are also used for other conditions, including coronary heart disease, where the drug reduces the formation of scar tissue following balloon angioplasty (Raja et al., 2006; Tanimoto et al., 2007). The taxol-eluding stents (TAXUS® Express2®, Boston Scientific, Natick, MA, USA), which received FDA approval in 2003/2004, have been shown to significantly decrease the risk of in-stent restenosis due to neointimal hyperplasia compared to bare-metal intracoronary stents. Due to the slow release of a cytostatic dose of taxol over an extended period, the drug reduces the neointimal growth after stent deployment. Assuming that a growing number of coronary heart decease cases will be treated with cardiovascular stents, the demand for taxol may increase even further (Lasala et al., 2006; Htay and Liu, 2005).

Supply has been the major challenge throughout the development of taxol. Taxol is too complex to synthesize economically from first principles and difficult and expensive to isolate from natural sources. Total synthesis provides a maximum yield of 2% taxol, which makes the production by this means commercially unfeasible (Danishefsky et al., 1996; Holton et al., 1994a, b; Nicolaou et al., 1994, reviewed in Xiao et al., 2003). Taxol makes up only a minor proportion of the total taxoid content of *Taxus* trees, and some species such as *Taxus baccata* (the European yew tree), produce hardly any taxol at all and only late precursors, such as Baccatin III (Nadeem et al., 2002). Extraction and purification are difficult and expensive because of these low yields; the commercial isolation of 1 kg of taxol from *T. brevifolia* requires the bark of 2000 - 3000 very slow-growing trees (Croom, 1995; Hartzell, 1991; Suffness, 1995).

The major current source of taxol and taxotere is semisynthesis (Holton et al., 1995). The late precursors

Baccatin III and 10-deacetylbaccatin III can be isolated from yew needles without killing the trees and can be modified with synthesized side chain molecules to arrive at the desired products. This production system still relies on yew trees for precursor molecules and therefore depends on epigenetic and environmental factors. An alternative production strategy is the use of *Taxus* cell suspension cultures, obtained from the species *T. brevifolia* (Gibson et al., 1993), *T. baccata* (Srinivasan et al., 1995) and *T. canadensis* (Ketchum et al., 1999). These cell cultures produce biomass faster than *Taxus* trees and can be grown under reproducible conditions. Under optimized culture conditions and following the induction of taxane production with methyl jasmonate, up to 23 mg/L/d of taxanes can be generated with a taxol content of 13 - 20% (Ketchum et al., 1999). These yields demonstrate the impressive biosynthetic capacity of *Taxus* cell cultures. However, sustaining such high rates of secondary metabolite production in plant cell culture is very difficult (Deus-Neumann and Zenk, 1984; Hall and Yeoman, 1987; Morris et al., 1989; Parr et al., 1990; Schripsema and Verpoorte, 1992). Since 2002, Bristol-Meyers-Squibb Inc. has switched its sourcing to plant cell culture-derived taxol (Ritter, 2004); however, most of the generic taxol is still derived from the semisynthetic production based on advanced taxoids isolated from collected plant material.

This gulf between demand and supply is the biggest challenge in the clinical application of taxol. This has driven research into new production strategies, such as metabolic engineering of the yeast *Saccharomyces cerevisiae* (Jennewein et al., 2005; DeJong et al., 2006; Engels et al., 2008) and the investigation of natural microbial producers, i.e., endophytic fungi. However, metabolic engineering of yeast for the total biosynthesis of taxol or other advanced taxoids is extremely complex and still in its infancy. Today, the total fermentation of taxadiene has been achieved in significant amounts in *S. cerevisiae* (Engels et al., 2008). Nevertheless the establishment of recombinant microorganisms, like yeast, offers great perspectives not only for the production of taxol but also for other complex natural products and derivatives thereof (Chang and Keasling, 2006).

THE TAXANE BIOSYNTHETIC PATHWAY

Taxol is a highly functionalized diterpenoid compound based on the common taxoid skeleton taxa-4(5),11(12)-diene (Koepp et al., 1995; Hezari et al., 1995). This is formed in the first committed step of the pathway from the universal diterpene precursor geranylgeranyl diphosphate. The backbone is then modified by several cytochrome P450-dependent monooxygenases and acyltransferases to yield either taxol or other taxoid compounds.

Since only a few of >350 known taxoid structures have known pharmacological properties (Kingston and Baloglu, 1999; Itokawa, 2003), it is essential to understand the regulation of this biosynthetic pathway to increase flux towards the desired compounds (Ketchum et al., 2003). After initial cyclization, there are probably many branch points that result in the great diversity of taxoid structures, e.g., 14β-hydroxy taxoids and 13-acetyl derivatives. These compounds may play a role in plant defense (Daniewski et al., 1998) as antibiotics (Young et al., 1992; Elmer et al., 1994) or toxins to discourage mammal herbivory (Ogden, 1988).

The biosynthesis of taxol from geranylgeranyl diphosphate is thought to involve 19 enzymatic steps and can be divided into several discrete processes (Jennewein et al., 2004b). The formation of the taxa-4(5),11(12)-diene backbone is followed by a sequence of eight hydroxylation reactions that require atmospheric oxygen (Eisenreich et al., 1998). This indicates that the reactions are catalyzed by cytochrome P450-dependent monooxygenases, which is also typical for many mono-oxygenation reactions in secondary metabolic pathways (Schuler, 1996). Floss and Mocek (1995) proposed the order to be C5 and C10, followed by C2 and C9, then C13 and C7 and finally C1 late in the pathway, based on the hydroxylation pattern of known isolated taxoids. Three of these hydroxyl groups are further acylated, including two acetylations and one benzoylation, although the timing of these reactions is not clear (Walker et al., 2002a). Advanced taxoids, such as Baccatin III, require further oxidation of the hydroxyl group at C9 and formation of the oxetane ring at C4,5. The last steps involve attachment of a β-phenylalanoyl side chain at C13 followed by 2'-hydroxylation and *N*-benzoylation. Related reactions lead to the *N*-tigloyl and *N*-hexanoyl derivates cephalomannine and Taxol C (Baloglu and Kingston, 1999).

We will discuss all steps in the pathway, mentioning the genes, enzymes and regulatory and evolutionary aspects where known. Progress in this research area was last briefly reviewed in 2006 (Croteau et al., 2006; Kaspera and Croteau, 2006).

PRIMARY METABOLISM AND PRECURSOR SUPPLY

The taxane core is derived via the plastidial 2-C-methyl-D-erythritol phosphate (MEP) pathway (Eisenreich et al., 1996), in which isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal precursors of all terpenes, are built from pyruvate and glyceraldehyde-3-phosphate through the intermediate 1-deoxy-D-xylulose-5-phosphate (DXP). This process has been reviewed (Rohmer, 1999; Eisenreich et al., 2001; Kutzuyama and Seto, 2003). A *T. cuspidata* plant cell culture cDNA library yielded expressed sequence tags (ESTs) encoding each of the seven enzymes involved in the plastidial pathway (Jennewein et al., 2004b).

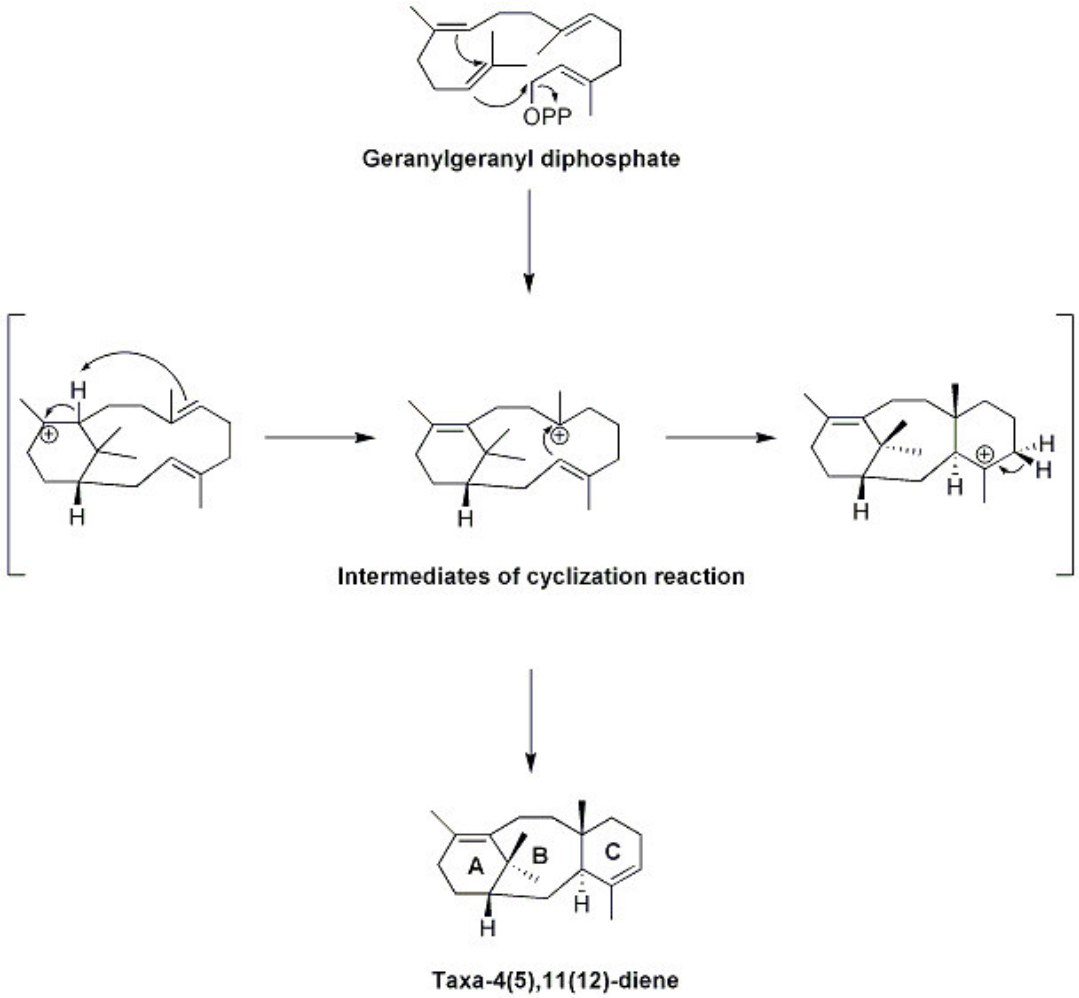

Figure 2. Mechanism of the cyclization of geranylgeranyl diphosphate, catalyzed by taxadiene synthase. This process begins with the formation of the A-ring, followed by proton migration that promotes the closure of rings B and C. Finally, the main product taxa-4(5),11(12)-diene is built through elimination at C5.

Like all diterpenoids, taxanes are based on geranylgeranyl diphosphate, which is derived from one molecule of DMAPP and three molecules of IPP via head to tail condensation. *Taxus* geranylgeranyl diphosphate synthase (GGPPS) was first isolated by the Croteau group from *T. canadensis* cells and later by the Verpoorte group from *T. baccata* cells (Hefner et al., 1998; Laskaris et al., 2000), and the protein was characterized as a typical prenyltransferase. The cDNA clone isolated by Croteau's group encoded a 32-kDa protein that assembled into a functional ~60-kDa homodimer. As expected, the amino acid sequence included a plastidial transit peptide. To examine the influence of GGPPS on the taxoid pathway, time course transcription profiling was carried out and showed that presumably neither constitutive nor induced GGPPS is rate limiting for taxol synthesis (Hefner et al., 1998). In the *T. cuspidata* cDNA library (Schoendorf et al., 2001), ESTs representing GGPPS were quite abundant, representing 1.7% of the clones (Jennewein et al., 2004b).

COMMITTING STEP – TAXA-4(5),11(12)-DIENE SYNTHASE (TDS)

The committing step in taxoid synthesis is the cyclization of geranylgeranyl diphosphate to taxadiene, catalyzed by taxadiene synthase (TDS) (Hezari et al., 1995) (Figure 2). Attempts to isolate TDS from *Taxus* trees yielded an enzyme with a molecular mass of ~79 kDa (Hezari et al., 1995). Its properties were similar to other plant terpene synthases, such as a relatively low K_m and Mg^{2+} as the cofactor, but unusually the optimum pH was 8.5. The main product of the enzyme was confirmed as taxa-4(5),11(12)-diene (Koepp et al., 1995), even though chemical analysis predicted preferential formation of the

4(20)-11(12)-isomer (Harrison et al., 1966, Guéritte-Voegelein et al., 1987). A 2586-bp cDNA clone encoding a 98-kDa pre-protein was isolated by a homology-based PCR cloning strategy (Wildung and Croteau, 1996). The corresponding enzyme contained an *N*-terminal plastidial targeting sequence, which was cleaved after import into the plastid, although the exact length of this sequence remains unclear. Heterologous expression of pseudomature, *N*-terminally truncated TDS variants in *E. coli* have indicated a sequence length of up to 79 amino acid residues (Williams et al., 2000b). Further analysis revealed features typical of plant terpene synthases, such as a DDXXD-motif responsible for cofactor binding, a conifer diterpene internal sequence domain and a glycosyl hydroxylase-like domain (Trapp and Croteau, 2001). In feeding experiments with the natural substrate GGPP, the enzyme produced 94% taxa-4(5),11(12)-diene, 5% taxa-4(20),11(12)-diene, 1% verticillene and trace amounts of the tentatively identified 3(4),11(12) isomer (Williams et al., 2000b).

Several investigations have focused on the mechanism of the TDS catalyzed cyclization reaction (Lin et al., 1996; Williams et al., 2000b; Jin et al., 2005a, b). The proposed mechanism for the cyclization reaction is shown in Figure 2. The reaction starts with ionization of the all-trans geranylgeranyl diphosphate ester at the C1 position, which promotes carbon-carbon bond formation between the C1 and C14 position of the substrate. This is followed by closure of the A-ring via a re-face attack at C10, yielding the 12-verticillyl cation intermediate. Intramolecular 11α to 7α proton transfer then generates 1S-verticillene (Williams et al., 2000b). The bound intermediate is rapidly reprotonated at the C7 position, via the same enzyme base responsible for the earlier deprotonation (Lin et al., 1996), to initiate transannular cyclization to generate the taxenyl cation. Finally, deprotonation at the β-*face* of C5 of the 4-taxenyl cation yields the endocyclic 4,5 double bond (Jin et al., 2005a). Minor pathways involve proton abstraction at the C20 and C3 α positions of the 4-taxenyl cation, yielding taxa-4(20),11(12)-diene and taxa-3(4),11(12)-diene, respectively (Jin et al., 2005a). Incubations of TDS with related macrocyclic diterpenes, cemberene A and verticillene, did not result in taxa-4(5),11(12)-diene (Lin et al., 1996), suggesting that partially cyclized intermediates remain tightly bound to the active site and no exchange with exogenous additives can occur during the catalytic cycle. A possible alternative cyclization mechanism of TDS in which the C-ring is formed first was ruled out based on the lack of incorporation of deuterium-labeled 2,7-*cyclo*-geranylgeranyl diphosphate into taxadiene (Williams et al., 2000b).

As with GGPPS, time course experiments were carried out with induced *Taxus* cell cultures. This showed that no taxadiene and no early intermediates accumulated, indicating that the cyclization reaction is very slow, although taxadiene synthase does not seem to be the rate limiting factor (Koepp et al., 1995; Hefner et al., 1996; Hezari et al., 1997).

TAXOID HYDROXYLASES

The first investigations focusing on the hydroxylation of the taxadiene core were carried out with cell-free extracts of *T. brevifolia* and *T. cuspidata* (Hefner et al., 1996; Lovy Wheeler et al., 2001). Using microsomal preparations of *T. brevifolia* stem and plant cell cultures, the first oxygenation reaction of taxadiene was identified as 5□-hydroxylation (Hefner et al., 1996). Characterization of the oxygenase activity could be conclusively identified by carbon monoxide inhibition and the reversal of enzymatic activity by irradiation with blue (450 nm) light and the oxygenase as a cytochrome P450-dependent monooxygenase (Hefner et al., 1996). Further biochemical studies using 5α-hydroxy-taxadiene and its acetate ester identified the following oxygenation reactions, 13α-hydroxylation and 10β-hydroxylation, respectively. Thus, using 5α-hydroxy-taxadiene and 5α-acetoxy-taxadiene as substrates in the microsomal preparations, 5α,13α-dihydroxy-taxadiene and 5α-acetoxy-10β-hydroxy-taxadiene were yielded, respectively (Figure 3) (Wheeler et al., 2001). The predominant roles of cytochrome P450s in catalyzing oxidation and hydroxylation reactions in plant secondary metabolic pathways are well established; however, purification of the enzymes from the plant microsomal fractions often proved difficult. Thus, to circumvent the obstacle of purification of the individual cytochrome P450 hydroxylases involved in taxol and taxoid biosynthesis, a molecular biology approach based on gene cloning, heterologous functional expression and enzyme functional testing was used. For the cloning of potential cytochrome P450 genes involved in the taxol biosynthesis, three complementary cloning approaches were applied. Taxol biosynthesis in plant cell culture is significantly increased in response to methyl jasmonate exposure (Yukimune et al., 1996). Using a differential display protocol for the specific cloning of cytochrome P450 genes (Schopfer and Ebel, 1998) and methyl jasmonate-elicited and nonelicited *T. cuspidata* cell cultures, several methyl jasmonate-induced cytochrome P450 partial clones were isolated, from which full-length cDNA clones were then obtained by traditional cDNA library screening (Schoendorf et al., 2001). This approach resulted in the isolation of the taxoid 10β-hydroxylase (Schoendorf et al., 2001) and 13α-hydroxylase (Jennewein et al., 2001). An alternative approach for the isolation of cytochrome P450 genes of the taxol biosynthetic pathway took advantage of highly conserved sequence regions of the cytochrome P450s (Holton and Lester, 1996). Using degenerate PCR primers designed

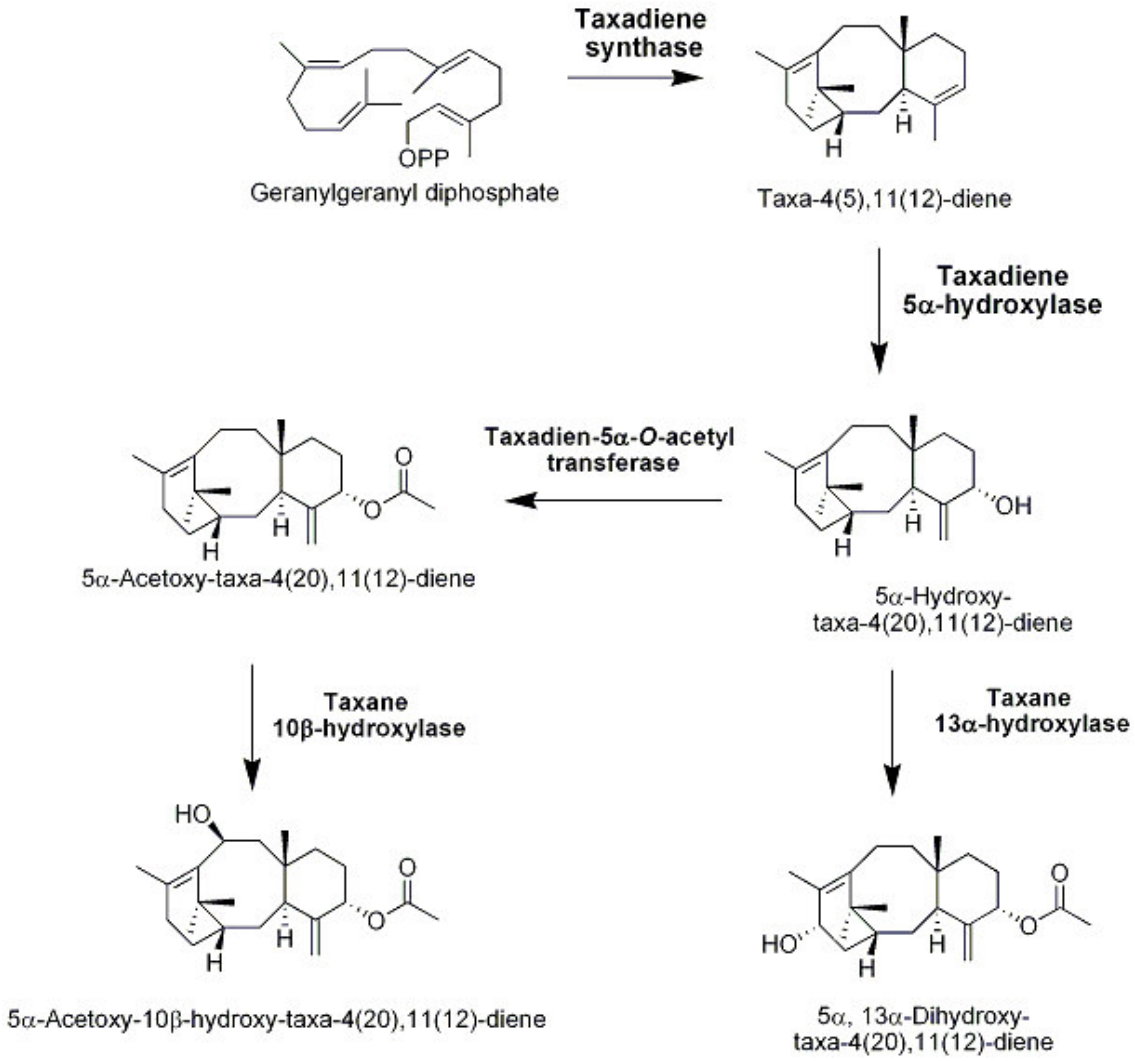

Figure 3. Early taxoid biosynthetic reaction steps identified by enzymatic assays of purified enzymes or microsomal fractions obtained from *Taxus* plant and plant cell culture.

from two highly conserved domains (the PERF motif and the heme-binding motif), several cytochrome P450 partial sequences could be isolated, from which the full-length clones were obtained also by traditional cDNA library screening (Jennewein et al., 2004a). In addition to the specific cloning of *Taxus* cytochrome P450 genes, random sequencing of a cDNA library from methyl jasmonate-induced *T. cuspidata* cell culture identified additional cytochrome P450 clones (Jennewein et al., 2004b). With the three chosen approaches, nearly 30 very similar (similarity >70%) candidates for cytochrome P450 genes with potential relevance to taxoid biosynthesis were obtained and most of the cloned genes were identified by all three approaches. The biosynthesis of taxol involves approximately nine oxygenation reactions, thus implying significant redundancy in hydroxylase functions. This redundancy in function was already encountered by the isolation of two different cytochrome P450 genes that encode a taxoid 10β-hydroxylase (Schoendorf et al., 2001; Jennewein et al., 2004b) and several very closely related sequences that code for taxoid 2α-hydroxylase (Chau and Croteau, 2004). The obtained *Taxus* cytochrome P450 genes were then functionally co-expressed with a NADPH: cytochrome P450 reductase either in the *Spodoptera frugiperda* baculovirus insect cell expression system (Jennewein et al., 2001) or in *S. cerevisiae* (baker's yeast) (Schoendorf et al., 2001; Jennewein et al., 2003). The insect cell expression system proved to be superior with regards to microsomal fraction preparation, whereas the yeast-based expression offered the advantage of using the more simple method of *in vivo* feeding of the radioactively labeled taxoid substrates for functional testing of the heterologous expressed cytochrome P450 genes.

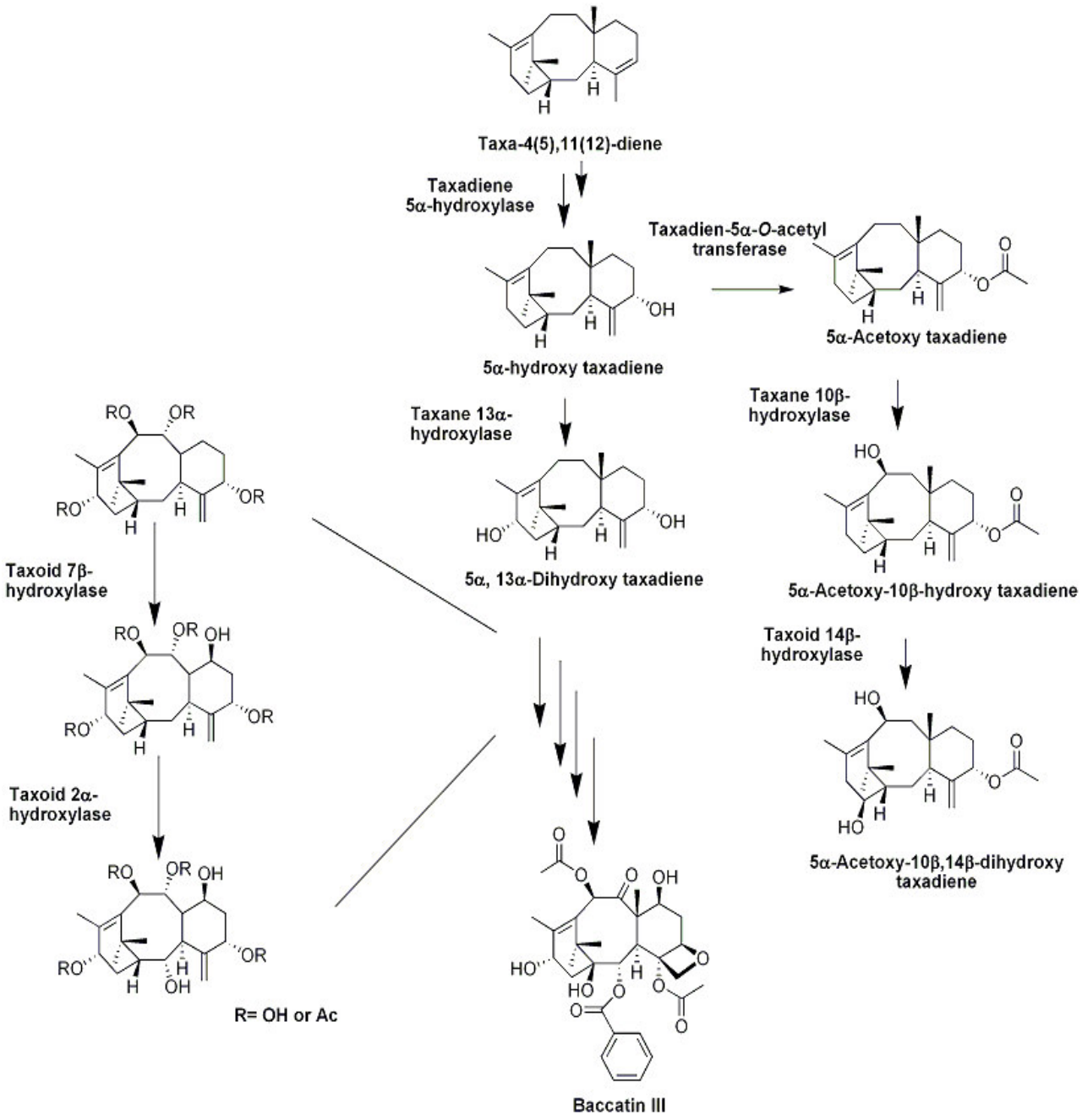

Figure 4. P450 monooxygenase-mediated hydroxylations of the taxa-4(5),11(12)-diene backbone lead to baccatin III. This process includes the early modifications at C5, C10 and C13, the C14 hydroxylation to major side products and the two modifications at C7 and C2, which are thought to be important in the main taxol biosynthetic pathway.

This approach of heterologous expression of cloned cytochrome P450 genes and functional testing of the expressed enzymes resulted in the identification of the taxoid 2α-, 5α-, 7β-, 10β-, 13α- and 14β-hydroxylases (Figure 4). All of the isolated sequences were very similar to each other, but more distantly related to other cytochrome P450 genes.

The hydroxylation steps of taxol biosynthesis can be divided into early, intermediate and late reactions. In microsomal fractions of *T. cuspidata,* six hydroxylations to taxadien-hexaol occurred under standard assay conditions (Wheeler et al., 2001). The first reaction is the conversion of taxa-4(5),11(12)-diene to 5α-hydroxy-taxa-4(20),11(12)-diene (Hefner et al., 1996). A cDNA encod-

ing the corresponding taxoid 5α-hydroxylase was isolated by homology and heterologous expression showed that the cytochrome P450 enzyme utilized taxa-4(5),11(12)-diene as well as taxa-4(20),11(12)-diene as substrates (Jennewein et al., 2004a). The catalytic mechanism involves an unusual allylic transposition of the 4,5- or 4,20-double bond to a delocalized radical intermediate, followed by hydroxylation at C5 and formation of the 4,20-double bond. Like many other early intermediates, the 5-hydroxylated taxadiene is found only in trace amounts in *Taxus* extracts (Ketchum and Croteau, 2006; Hefner et al., 1996). The compound is likely to be converted rapidly by the subsequent hydroxylases and acyltransferases. The next modifications are probably C13 and C10 hydroxylations. The cDNA clones of the taxoid 10β- and 13α-hydroxylases were isolated by differential display (Schoendorf et al., 2001; Jennewein et al., 2001) and the corresponding enzymes were shown to use both 5α-hydroxy-taxadiene and 5α-acetoxy-taxadiene but with opposite substrate selectivities. Although 5α-hydroxy-taxadiene and 5α-acetoxy-taxadiene are substrates for both enzymes, the former is favored for 13-hydroxylation whereas the later is more likely to be modified at C10. Together with results from cell feeding studies using the relevant intermediates (Ketchum and Croteau, 2006), it is likely that a bifurcation occurs early in the taxol biosynthesis pathway, one branch leads to taxol and the other to alternative taxoids (or perhaps to taxol via a different route). Isolation of a clone encoding taxoid 14β-hydroxylase by screening transformed yeast cells containing P450 candidates (Jennewein et al., 2003) supports the hypothesis of diversification at this early stage. The enzyme converts 5α-acetylated derivatives to 14β-hydroxylated taxadienes, demonstrating the importance of acyltransfer reactions, such as acetylation at C5 (Walker et al., 1999), for the ultimate distribution of taxoid products. 14β-hydroxylated compounds, e.g. taxuyunnamine C, are prominent compounds isolated from *Taxus* cell cultures, which is consistent with the abundance of the corresponding cDNA clones in the induced *Taxus* cDNA library (Jennewein et al., 2004b). This early branch point has been the subject of intensive studies. Feeding experiments in *Taxus* cell cultures with the radio-labeled substrates taxa-4(5),11(12)-diene, 5α-hydroxy-taxa-4(20),11(12)-diene, 5α,10β-dihydroxy-taxa-4(20),11(12)-diene, 5α-acetoxy-taxa-4(20),11(12)-diene and 5α-acetoxy-10β-hydroxytaxa-4(20),11(12)-diene clearly showed the influence of acetylation at C5 on the conversion to C14 taxoids (distribution of C13:C14 was 47:33 with 5α-acetoxy-taxa-4(20),11(12)-diene). However, hydroxylation at C10 directed the flux to C13 taxanes (75% C13 taxanes) (Ketchum et al., 2007).

C9-hydroxylation is also thought to be an early reaction in the pathway. *In vivo* studies in which yeast were fed 5α-hydroxy-taxadiene showed that one cDNA encoding a P450 candidate might represent a taxoid 9α-hydroxylase, but the product has yet to be confirmed by NMR (Croteau et al., 2006).

The analysis of intermediate oxygenation steps was much more difficult than the above mentioned reactions. This reflects the limited supply of substrates, first because they cannot be isolated from natural sources and second because of the unknown order of the reactions. Nevertheless, it was possible to identify the taxoid 2α- and 7β-monooxygenases (Chau and Croteau, 2004; Chau et al., 2004). Therefore, taxusin (5α, 9α, 10β, 13α-tetraacetoxy-taxa-4(20),11(12)-diene), a compound isolated from yew heartwood that is thought to be a dead-end metabolite rather than an intermediate in taxol synthesis (Koepp et al., 1995), was used as the test substrate. It was shown that both enzymes could operate sequentially, with 7β-hydroxylation probably followed by 2α-hydroxylation. By incubating microsomes with taxusin, the common hexaol (2α, 7β-dihydroxy taxusin) was formed. Furthermore, the enzymes demonstrated a preference for acetylated compounds, in particular the 5α-acetylation reaction promoted tight substrate binding, whereas modifications at the other positions (C13, C10 and C9) appeared less important (Chau et al., 2004). However, the conversion of highly functionalized compounds clearly indicated the role of 2α- and 7β-hydroxylases in the mid to late phases of the Taxol biosynthetic pathway.

Two oxygenation steps remain to be examined: the C1β-hydroxylase step and the C4β, C20-epoxydase step, which are responsible for the formation of an oxetane ring at C4 and C5. Both reactions are likely to be P450 mediated. The lack of substrates and the uncertain order of the reactions late in the pathway make it difficult to clarify this part of the pathway. The hydroxylation at C1 could be investigated using compounds like Baccatin I (lacking the 1β-hydroxyl but possessing the 4,20-epoxide) or 1β-dehydroxy-Baccatin VI. Examination of D-ring formation may be more difficult because several different reaction mechanisms are equally plausible. All implicate the progression of the 4,20-ene-5α-oxy functional group through the 4,20-epoxide derivative to the final oxetane (Floss and Mocek, 1995; Walker and Croteau, 1999, 2001) (Figure 5). The first possible mechanism involves rearrangement of the 4,20-epoxide by protonation and ring-opening of the epoxide, allowing the acetoxy group from C5 to migrate to C4 in the ring expansion process via a dioxonium ion intermediate (Gueritte-Voegelein et al., 1987; Giner and Faraldos, 2003). The other possibility is a transferase type mechanism catalyzed by an oxomutase and consisting of a nucleophilic attack by the C5-acetyl-group at C4 causing epoxide ring opening, followed by intramolecular migration of the acetoxy group resulting in the formation of the oxatane ring.

Another unknown step on the way towards Baccatin III

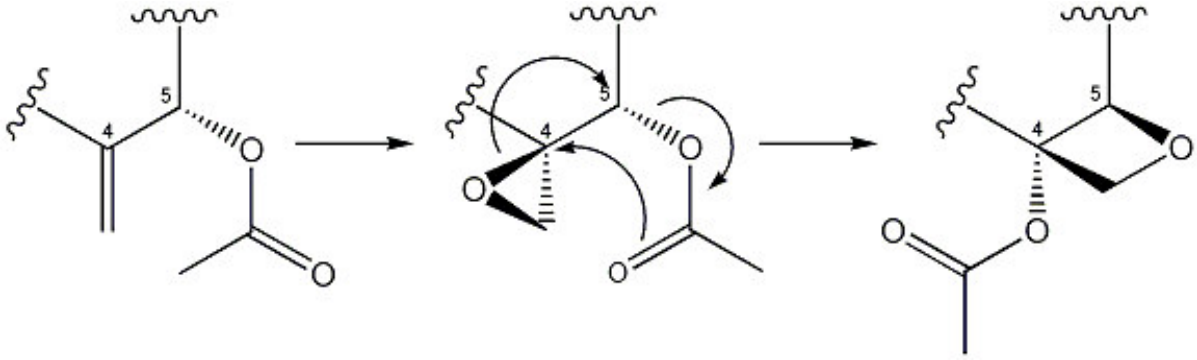

Figure 5. Synthesis of the oxetane ring, starting from 5α-acetoxy-taxa-4(20),11(12)-diene through the 4(20)-epoxy-intermediate.

is the oxidation of the hydroxyl group at C9. Reactions like this may be catalyzed, as hydroxylations, either by cytochrome P450 enzymes via the ketone hydrate as shown for other secondary metabolites (MacMillan and Beale, 1999) or by pyridine nucleotide-dependent dehydrogenases. *T. cuspidata* cells revealed many dehydrogenase candidates that have to be tested for C9-oxidation with 9α-dihydrobaccatin III derivatives.

With at least two monooxygenases still unknown and many *Taxus* cytochrome P450 genes undefined, the order of reactions in taxol biosynthesis and any corresponding phylogenetic analysis can only be regarded as approximate. It is reasonable to assume that the family of taxoid cytochrome P450-dependent monooxygenases evolved through gene duplication and divergence from a common ancestor (Pichersky and Gang, 2000), as suggested by the >70% similarity among the taxoid hydroxylases and much lower similarity to other plant-derived P450 monooxygenases (Jennewein et al., 2004b). This homology-functionality relationship is also observed in steroid biosynthesis, where hydroxylases with the same catalytic capability show up to 68% similarity, whereas those with different functions also have more diverse sequences (Feldmann et al., 2002; Kim and Tsukaya, 2002). Phylogenetic comparison demonstrates the tight coherence of taxoid cytochrome P450 enzymes and the very distant relationship to other plant cytochrome P450s (< 35%). Only catalytically similar enzymes, such as abietadienol/abietadienal oxidase from loblolly pine (Ro et al., 2005), are grouped together with taxoid P450s (Kaspera and Croteau, 2006).

It is possible that the similarity between individual enzymes and the parent sequence might indicate the order of oxygenations in taxol biosynthesis. Construction of a cladogram rooted at the C5α-hydroxylase (presumed to be the initial hydroxylation step) generated a series of branches matching the assumed order of reactions, starting with C5α-, followed by C13α- and C10β-hydroxylation. Furthermore, this analysis also indicates that C14 hydroxylation occurs early, at the taxa-4(20),11(12)-dien-5α-ol/acetate stage, constituting a major side branch in taxoid metabolism (Jennewein et al., 2003). The other known taxoid hydroxylases (C2, C7) were much more similar to each other (65%) than to the early pathway hydroxylases (only 55% similarity to 10β-hydroxylase), although they were placed nearer to 5α-hydroxylase (62%) than might have been anticipated based on the proposed order of reactions and the preferences of the 2α- and 7β-hydroxylases for highly functionalized substrates.

For clear evidence on the order of taxoid hydroxylation steps, it will be necessary to identify and characterize the missing monooxygenases. Therefore, it is necessary to get access to more highly functionalized (intermediate) taxoids, but due to the complexity of total chemical synthesis of taxoids and the inaccessibility from natural sources, the current unavailability represents a major obstacle in the functional assignment in the intermediate and late hydroxylation reactions. A possible solution for this limitation may be the biosynthesis of these intermediates in a heterologous system like yeast (Engels et al., 2008).

ACYLTRANSFERASES

Taxol contains four ester functional groups at C2 (benzonate), C4 (acetate), C10 (acetate) and C13 (*N*-benzoyl-3-phenylisoserinoyl) on the taxane core. Among the enzymes responsible for these reactions, the first to be investigated was the 5-*O*-acetyltransferase. Acetylation in this position is considered to be the progenitor of the rearrangement reaction leading to the oxetane ring of taxoids (Walker et al., 1999). After demonstrating activity in *T. canadensis* soluble protein extracts, the enzyme was partially purified and shown to be a 50-kDa acetyl-CoA-dependent transferase with a pH optimum of ~9.0, a low μM K_m value and selectivity for less functionalized taxanols (Walker et al., 1999). With the objective to isolate more acetyltransferases of taxol biosynthesis,

degenerate primers were designed according to a consensus protein sequence, obtained from an alignment of transacylase sequences of plant origin, and used to generate PCR probes with which then a *T. cuspidata* cDNA library were screened. This yielded 8 full-length cDNAs and 7 ESTs, leading to the identification of 15 acyltransferase-type genes (Walker et al., 2000; Jennewein et al., 2004b). Functional analysis in *E. coli* soluble protein extracts resulted in the identification of the taxadien-5α-ol-*O*-acetyltransferase, the taxoid-2α-*O*-benzoyl transferase, the 10β-*O*-acetyltransferase and two cDNA clones encoding enzymes involved in transferase reactions at the C13 side chain. All 15 candidates encoded proteins of ~50 kDa without *N*-terminal targeting peptides, indicating localization in the cytosol. The function of the core acylating enzymes, including regio- and substrate-specificity (acetyl-CoA is an inefficient donor for the 5α-transferase), was confirm-ed using appropriate test substrates (e.g., 2-debenzoyl-7,13-diacetylbaccatin III). The enzymes were also partially characterized (Walker and Croteau, 2000a, b).

Defining the order of acylation is even more difficult than for the monooxygenases because such modifications lead to an extremely large number of taxoid side products. As well as modifications that affect the amino group of the side chain (see later), many compounds are known that have different acylation patterns, thus providing multiple acylation sites and different substitutions (Baloglu and Kingston, 1999; Itokawa, 2003).

Furthermore, it is conceivable that some of these metabolites are true intermediates in taxol biosynthesis and involved in trafficking processes mediated by acetylation/deacetylation, which could greatly increase the number of steps and the complexity of the pathway. So it is probable that the remaining 10 transferase genes encode enzymes that catalyze the production of side branch metabolites or as yet undefined intermediates.

In order to explore the numerous *Taxus* acyltransferase candidates, polyhydroxylated taxoids were used as substrates (Chau et al., 2004b). This approach yielded one new transferase that could produce the C5-acetylated taxadiene. Comparison of this new taxadien-5α-ol-*O*-acetyltransferase and the known enzyme showed that they had the same activity for C5-acetylation and were both able to functionalize highly hydroxylated taxoids. Surprisingly they showed different regioselectivities, one for C9 and C10 and the other for C5 and C13. These results indicated that the acyltransferases are not substrate-specific and only moderately region-specific. The acylation position appears to depend very much on the substitution pattern of the metabolized precursor (Chau et al., 2004b). Although the precise timing of C5 acetylation is not completely clear, it is still probable that the step occurs early in the pathway and is somehow influenced by division into C13 and C14 taxoid syntheses (Ketchum et al., 2007).

Like the cytochrome P450-dependent monooxygenases, the taxoid acyltransferases are very similar to each other (> 65%) and probably have also evolved from a common ancestor by gene duplication and divergence. This explains the different regio- and substrate specificities and also may explain why the enzymes can accept more than one substrate. The relative phylogenetic placement of the transferases (Jennewein et al., 2004b) is consistent with current data that set taxadien-5α-ol-*O*-acetyltransferase before taxoid-2α-*O*-benzoyl transferase and 10β-*O*-acetyltransferase (which is likely to convert 10-deacetylbaccatin III to baccatin III) prior to side chain assembly. Further feeding studies with a greater variety of precursors will be required to gain more insight into the characteristics and selectivities of taxoid acyltransferases.

C13 SIDE CHAIN ASSEMBLY

The assembly of the side chain at C13 is probably the last modification of the taxane core. The β-phenylalanoyl-type side chains were shown to be formed from α-phenylalanine through the activity of an aminomutase (Leete and Bodem, 1966; Platt et al., 1984) (Figure 6). Feeding studies with *Taxus* cells demonstrated that the *N*-benzoyl-3'-phenylisoserinoyl side chain of taxol also originates from α-phenylalanine metabolism (Fleming et al., 1994). The mutase activity responsible for this committed step in side chain biosynthesis was first observed in *Taxus* stem extracts by Walker and Floss (1998). Further feeding studies with baccatin III, the supposed substrate, were performed with either β-phenylalanine or phenylisoserine, showing that both molecules were incorporated, although the unbenzylated amino acid was three-times more efficient as a substrate. However, *N*-benzoyl phenylisoserine was not a suitable substrate, indicating the formation of β-phenylalanoyl- or phenylisoserine-baccatin III prior to *N*-benzoylation. These results do not indicate the timing of 2'-hydroxylation. The acceptance of phenylisoserine indicates that the reaction occurs before the attachment of the chain, although no amino acid hydroxylase activity has been detected thus far (Silverman, 2000). On the other hand, *Taxus* microsomes can catalyze the conversion of β-phenylalanoyl-baccatin III to phenylisoserine-baccatin III (Long and Croteau, 2005), indicating the possibility of hydroxylation comparable to oxygenation of the taxane core structure. The undefined cytochrome P450 cDNAs from the *Taxus* library therefore remain possible candidates for a taxoid 2'-hydroxylase. A taxoid aminomutase cDNA was isolated from the *T. cuspidata* library and expressed functionally in *E. coli* (Walker et al., 2004). The cDNA encoded a 76.5-kDa enzyme that did not require cofactors and showed the typical enzyme motifs (Walker et al., 2004). The se-

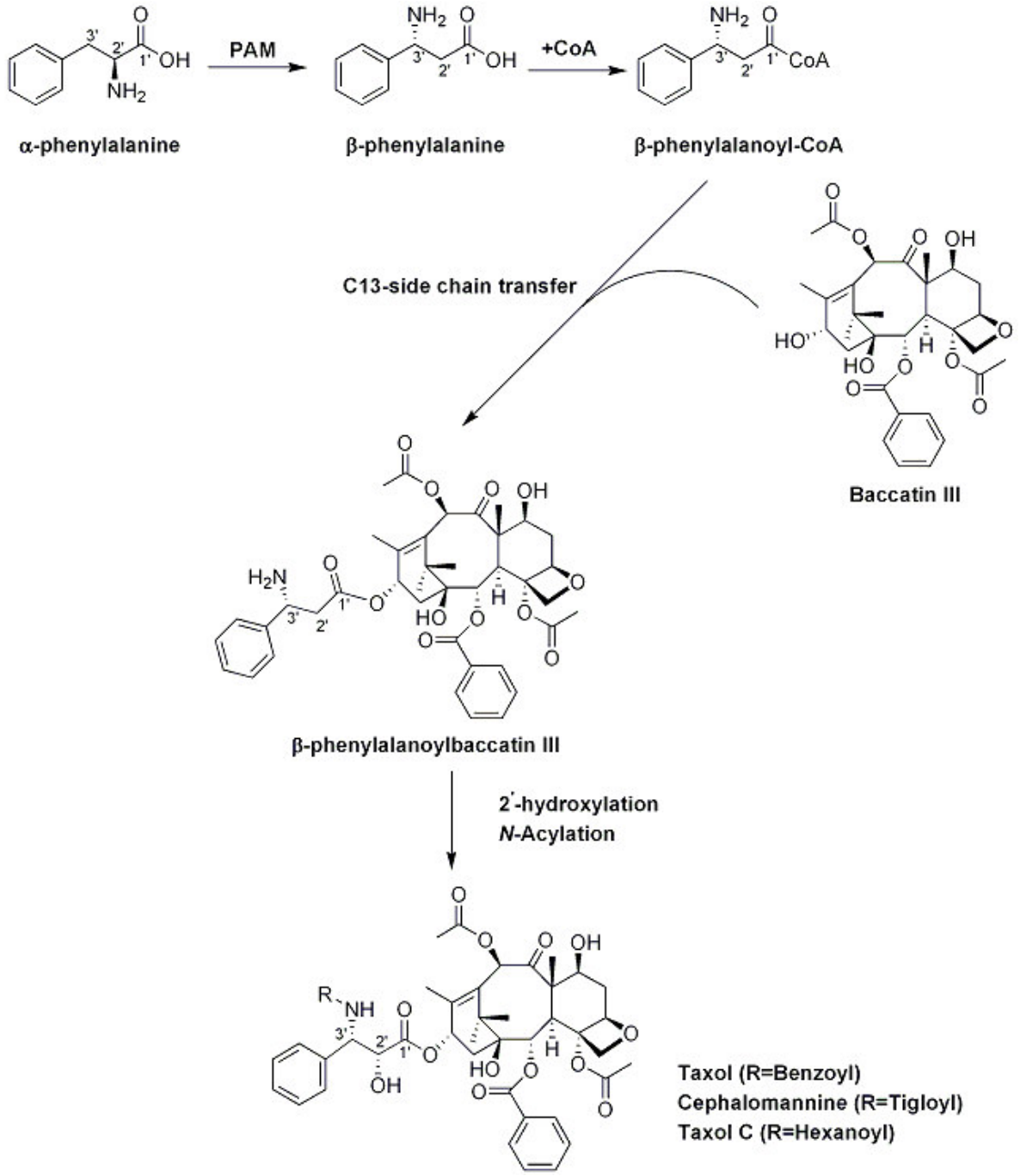

Figure 6. Biosynthesis of □-phenylalanoyl-CoA, the precursor of the taxane C13 side chain, followed by transfer to Baccatin III forming β-phenylalanoylbaccatin III, which is finally 2'-hydroxylated and *N*-acylated to form taxol (*N*-benzoyl) or derivatives such as cephalomannine (*N*-tigloyl) and taxol C (*N*-hexanoyl).

quence was nearly identical to that of clones isolated from *T. chinensis* using an alternative genetic approach (Steele et al., 2003). The side chain assembly is catalyzed by C13 propanoyl-CoA transferase (Walker et al., 2002b). The full-length cDNA clone, encoding a 50.5-kDa soluble protein, was functionally expressed in *E. coli* and found to transform Baccatin III and α-phenylalanoyl-CoA to the expected product, *N*-debenzoyl-2'-deoxytaxol. The transferase appeared to be highly regioselective for esterification at C13, but the specificity for the CoA donor was not absolute. As observed in experiments with total *Taxus* microsomes, the recombinant enzyme accepted

α-phenylalanoyl-CoA (V_{rel} = 100) and phenylisoserinoyl-CoA (V_{rel} = 40), which shows that the reaction needs a free 3-amino group. Hence, *N*-benzoylation is the last step in the Taxol biosynthetic pathway.

A side chain *N*-benzoyl transferase was identified by functional screening of the set of acyltransferases obtained from *T. cuspidata*. Using *N*-debenzoyl-2'-deoxytaxol as a test substrate, a full-length cDNA encoding a 49-kDa protein was isolated and the protein was shown to have a K_m value for both substrate and co-substrate of ~400 µM (Walker et al., 2002a).

In order to determine whether *N*-debenzoyl-2'-deoxytaxol or *N*-debenzoyltaxol was the natural substrate for taxoid *N*-benzoyl transferase, *N*-debenzoyltaxol was synthesized and the enzyme kinetics for both compounds were determined (Long et al., 2008). The efficiency of benzoyl-CoA transfer to the 2'-hydroxylated substrate was shown to be double that of unsubstituted substrate, indicating that *N*-debenzoyltaxol is the preferred precursor for taxoid *N*-benzoyl transferase. In this context, selectivity for the CoA co-substrate was also tested. Only taxoids varying with respect to 3'-*N*-substitution are observed in *Taxus* cell cultures, e.g., Taxol (*N*-benzoyl-3'-phenylisoserinoyl), cephalomannine (tigloyl) and Taxol C (hexanoyl) (Kingston and Baloglu 1999). The taxoid *N*-benzoyl transferase was found to be highly selective for benzoyl-CoA and did not convert any other substrate (Long et al., 2008). This indicates that some of the still uncharacterized acyltransferase candidates might correspond to enzymes that facilitate the diversification of taxoid composition at this last step of the biosynthetic pathway.

ENDOPHYTIC FUNGI – A POTENTIAL NEW SOURCE FOR TAXOL

The search for new secondary metabolites focuses on organisms that inhabit unique biotopes, since this is more likely to indicate the production of unique natural products. Endophytes are bacteria and fungi that live within plants, and because their environments are unique, they are considered a wellspring of novel secondary metabolites with significant potentials for medical use. They are defined as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Bacon and White, 2000). The number of endophytic species is unknown, but may exceed one million, providing an extremely large pool of biological and hence biochemical diversity (Dreyfuss and Chapela, 1994). Endophytic fungi have thus far yielded compounds with a range of properties, including antibiotics, antivirals, antioxidants, antidiabetic agents, immunosuppressive compounds, insecticidal products and anticancer agents (Strobel and Daisy, 2003; Strobel et al., 2004). Of particular relevance to this review are those endophytic fungi that produce the same natural products as their host plant species, the most prominent example being taxol. Studies of endophytic fungi from *T. brevifolia* in the 1990s led to the isolation of *Taxomyces andreanae* (Stierle et al., 1993), which could produce taxol and secrete it into the growth medium, allowing extraction with organic solvents. Taxol production was confirmed through the use of immunological assays with a monoclonal anti-taxane antibody, by thin layer chromatography and by HPLC-mass spectrometry to derive structural information, showing that the fungal product was identical to that produced in plants (Stierle et al., 1993). Further investigation involving feeding studies with ^{14}C-labeled precursors has confirmed the synthesis of taxol by this fungus.

The collection of endophytic fungi from different yew species all over the world resulted in the isolation and identification of numerous taxol-synthesizing fungi. *Pestalotiopsis* spp., such as *Pestalotiopsis microspora* found on *T. wallichiana,* were found not only on yews but also on cypress trees, which do not produce taxoids (Strobel, 2002; Strobel et al., 1996). This unexpected discovery led to an enlargement of the search for taxol-producing microbes beyond *Taxus* species. *Pestalotiopsis guepini* found on *Wollemia nobilis* and *Seimatoantlerium tepuiense* found on *Maguireotha-mnus speciosus* are examples of taxol-producing endophytes from sources outside of the *Taxus* genus (Strobel et al., 1997, 1999). More recently, many more endophytic fungi have been isolated from *Taxus* species in Asia, including *Fusarium solani* from *T. celebica* (Chakravarthi et al., 2008) and the so-called fungus BT2 and 12 unnamed fungi from *T. mairei* (Guo et al., 2006; Zhou et al., 2007). In China, *Sporormia minima* and a fungus from the genus *Trichothecium* have been isolated from *T. wallichiana* (Wang et al., 2000; Shrestha et al., 2001) and another three endophytes have been obtained from *T. x media* and *T. yunnanensis* (Zhang et al., 2008).

These data show that taxol-producing fungi may be found all over the world and one logical explanation for this is that taxol inhibits the growth of pathogenic fungi, such as *Pythium* sp and *Phytophthora* sp., which benefits the host (Young et al., 1992). However, it is not clear why such fungi are found so often on plants that already produce taxoids (and that do so at levels much higher than any fungus reported thus far). This raises questions about the evolutionary origin of taxol biosynthesis.

Comparative analyses of corresponding plant and fungal enzymes reveal significant differences in sequence, structure and function. For example, the phylogenetic comparison of several plant terpene cyclases with trichothecene synthase from *F. sporotrichioides* shows a number of differences. Whereas plant cyclases share certain characteristics, such as the conserved relative positions of the catalytic DDXXD motif, and can be divid-

ed into classes according to such features, the same is not possible for fungal terpene cyclases. The analysis of a fungal diterpene cyclase reveals the evolutionary gulf: a diterpene synthase from *Phomopsis amygdali* is able to synthesize GGPP from the universal precursors IPP and DMAPP and then directly catalyses the cyclization to the diterpene backbone (Toyomasu et al., 2007). Examples from other metabolic pathways confirm this very distant relationship between plant and fungal enzymes. Gibberellins are found in plants and fungi and the enzymes catalyzing the initial synthesis step and the formation of *ent*-kaurene, are very different. In plants, the reaction is catalyzed by two enzymes with copaly diphosphate as an intermediate (Olszewski et al., 2002), whereas in fungi, such as *Gibberella fujikuroi*, there is a single enzyme that catalyses the reaction directly from geranylgeranyl diphosphate to *ent*-kaurene (Tudzynski and Hölter, 1998).

Gibberellins are synthesized in nearly all plants because of their central role as growth hormones. Therefore, independent evolution of the biosynthetic pathway in plants and microorganisms is plausible. This is unlikely in the case of taxol because it is a niche compound and tends to be produced by plants and fungi living commensally. Both plants and fungi could be considered as the origin of the pathway, but the complete and independent evolution of such a complex pathway in two different kingdoms is improbable. Therefore, we must consider the possibility of lateral, trans-kingdom gene transfer in the co-evolution of taxol biosynthesis. The plant is unlikely to be the donor of the pathway because the genes in the yew are most likely scattered throughout the genome and a series of coordinated vertical gene transfers would be required to recapitulate the pathway in the fungus. Another possibility is that an ancient precursor of the plant terpene cyclase moved to the fungus by vertical transfer and this independently gave rise to the whole pathway by divergent evolution.

Gene transfer from a fungus to the host plant is more likely if the genes are clustered, which appears to be a mechanism by which fungi prevent the loss of genetic information (Walton, 2000). Four examples are known concerning diterpene synthesis. First, gibberellin synthesis in *G. fujikuroi* involves a copalyl synthase/kaurene synthase gene, which is clustered with genes encoding a geranylgeranyl diphosphate synthase and three P450 monooxygenases (Tudzynski and Hölter, 1998). Second, aphidicholin synthesis in *Phoma betae* involves a gene cluster encoding a terpene cyclase, a geranylgeranyl diphosphate synthase and several cytochrome P450 monooxygenases, as well as a transcription factor and a transport factor (Toyumasu et al., 2004). Third, aflatrem biosynthesis in *Aspergillus flavus* also involves a cluster of genes encoding a terpene cyclase, a geranylgeranyl diphosphate synthase, several cytochrome P450 monooxygenases and transcription and transport factors (Zhang et al., 2004). Finally, two gene clusters for the biosynthesis of diterpenes were isolated from *Phomopsis amygdali*, including geranylgeranyl diphosphate synthases, terpene cyclases and several candidates for P450 monooxygenases (Toyomasu et al., 2008). These data suggest that taxol biosynthesis genes could also be clustered in fungi, providing a more plausible origin for the entire pathway in plants.

Although the possibility of a gene cluster for taxol biosynthesis in fungi is very interesting in the context of gene transfer and evolution, it could also provide a useful tool to identify missing genes and enzymes in the pathway (e.g., the enzyme responsible for the formation of the oxetane ring). Overall, investigating the genes for taxol biosynthesis in endophytic fungi will better our understanding of this pathway and provide information about trans-kingdom gene transfer and its impact on the evolution of secondary metabolism in general. The fruits of this research may lead to the development of fungal systems for the commercial production of taxol.

Conclusions

Taxol is widely used as an anticancer drug, but the challenging supply and demand balance has driven an extensive search for new sources to replace the yew tree *T. brevifolia*. Phytochemical analysis has shown that taxoids are strictly limited to the genus *Taxus* and some endophytic fungi. The evolutionary basis for such an elaborate biosynthetic pathway, which involves many enzymatic steps from different enzyme classes and (in plants) compartmentalization into the plastid, endoplasmic reticulum and cytosol remains unsolved. Many of the pathway's enzymes are now known, and the corresponding genes have been cloned and characterized. This basic research now opens the door to metabolic engineering for the improvement of taxoid synthesis, either by the direct modification of taxoid-producing cells (*Taxus* cell cultures, endophytic fungi) or by transferring the entire pathway to a heterologous but well-characterized surrogate, such as the baker's yeast *S. cerevisiae*. These exciting developments may well usher in a future in which for the first time we can consider the possibility of an unlimited supply of taxol and its related compounds.

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