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Feeding Cultured *Taxus* Cells with Early Precursors Reveals Bifurcations in the Taxoid Biosynthetic Pathway

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Abstract

Feeding studies with *Taxus* suspension cells employing labeled 5 α -hydroxytaxadiene and 5 α ,10 β -dihydroxytaxadiene, and the corresponding 5 α -acetate esters, demonstrated that acetylation at C5 of the monool precursor promotes the formation of 14 β -hydroxy taxoids, such as taxuyunnanine C, at the expense of 13 α -hydroxy taxoids, including Taxol and its congeners, but that the major bifurcation in taxoid biosynthesis, toward 13 α - or 14 β -hydroxy taxoids, occurs after 10 β -hydroxylation of the taxane core.

Keywords

Taxus media; *Taxus cuspidata*; Taxaceae; Yew; Taxadienol acetylation; Taxol; Taxuyunnanine C

1. Introduction

The anticancer drug Taxol (**1**) (generic name paclitaxel) is but one of the structurally more complex representatives of the approximately 400 defined taxoids (i.e., taxane diterpenoids) produced by *Taxus* (yew) species (Baloglu and Kingston, 1999; Itokawa, 2003), all of which are based on the unusual taxane (pentamethyl[9.3.1.0]^{3,8}tricyclopentadecane) skeleton, or rearrangement products of this tricyclic scaffold (Figure 1). The rationale for the biosynthesis of such a vast assortment of structurally diverse taxoids by *Taxus* species is unknown, although a role for these metabolites in defense seems likely for this long-lived plant genus (Odgen, 1988). A small number of these 400 or so taxoid metabolites are almost certainly relevant intermediates of the Taxol (**1**) biosynthetic pathway, which is considered to involve 19 discrete enzymatic steps from primary metabolism (Croteau et al., 2006), but most taxoids seemingly represent side-routes and metabolic dead-ends perhaps resulting as the consequence of promiscuous oxygenase and acyltransferase activities. It is clear that both intact *Taxus* tissues (Kikuchi and Yatagai, 2003) and derived cell cultures (Takeya, 2003) direct considerable precursor flux to the production of taxoids other than Taxol (**1**), and that any approach to improving the production yields of the drug and its useful immediate precursors must take into account these numerous and apparently diversionary taxoid biosynthetic pathways.

The committed step in the biosynthesis of Taxol (**1**) and other taxoids is the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (**2**) to taxa-4(20),11(12)-diene (**3**) to establish the taxane ring system (Koepp et al., 1995). The cyclization is then followed

by the first hydroxylation of the pathway at the C5 α -position, with allylic migration of the double bond, to afford the second established intermediate 5 α -hydroxytaxa-4(20),11(12)-diene (**4**) (Figure 1) (Hefner et al., 1996;Jennewein et al., 2004). An extended series of acylations and hydroxylations next occur that, along with an oxidation at C9 and oxetane (D-ring) formation, yield the advanced intermediate baccatin III, upon which the C13 α -O-side chain (*N*-benzoyl phenylisoserine) is constructed in several additional steps to yield Taxol (**1**) [for a recent review of Taxol (**1**) biosynthesis and molecular genetics, see (Croteau et al., 2006).]

Whereas this general description of the pathway is possible, the immediate reactions leading from 5 α -hydroxytaxadiene (**4**) on route to Taxol (**1**) are presently unclear, and *in vitro* biochemical studies allow for several possibilities involving acetylation at C5 followed by hydroxylation(s), or subsequent hydroxylation(s) followed by acetylation, as means of pathway progression (Lovy Wheeler et al., 2001;Walker et al., 1999). Thus, the cytochrome P450 taxoid 10 β -hydroxylase (which is presumed to catalyze the second pathway oxygenation step) competently mediates the regio- and stereo-specific 10 β -hydroxylation of both 5 α -hydroxytaxadiene (**4**) and 5 α -acetoxytaxadiene with the acetate ester preferred (Schoendorf et al., 2001), whereas the cytochrome P450 taxoid 13 α -hydroxylase (also considered to catalyze an early Taxol (**1**) pathway oxygenation step) also competently mediates the regio- and stereo-specific 13 α -hydroxylation of both alcohol and ester with the free alcohol as preferred substrate (Jennewein et al., 2001). Additionally, the cytochrome P450 taxoid 14 β -hydroxylase, also thought to operate early in the pathway and to direct the formation of side-route 14 β -hydroxy taxoids such as taxuyunnanine C (**5**) (Figure 1), utilizes principally the C5-acetate esters of 5 α -hydroxytaxadiene (**4**) and 5 α ,10 β -dihydroxytaxadiene as preferred substrates (Jennewein et al., 2003).

To gain a clearer understanding of the early pathway steps leading from 5 α -hydroxytaxa-4(20), 11(12)-diene (**4**) to Taxol (**1**) and its congeners, we conducted feeding studies with relevant early precursors using suspension cultured *Taxus* cells. It was of additional interest to explore the origins of the 14 β -hydroxy taxoids (taxuyunnanine C (**5**) and its relatives) in this *in vivo* experimental system (Ketchum and Croteau, 2006) in which the family of 14 β -hydroxy taxoids can constitute a substantial portion of the total taxoid mix (up to 30%) and thus represent a significant diversionary pathway (i.e., metabolites bearing the 14 β -hydroxy function almost certainly cannot proceed to Taxol (**1**)). It is of further note that the 13 α -hydroxy taxoids (Taxol (**1**) and its relatives) and the 14 β -hydroxy taxoids (taxuyunnanine C (**5**) and its relatives) constitute distinct and nearly mutually exclusive groups of metabolites, in that taxoids bearing both substituents (i.e., 13,14-vicinal oxygen functions) are of very rare occurrence in *Taxus* (Baloglu and Kingston, 1999;Itokawa, 2003). The results of the feeding studies described herein reveal that pathway bifurcations toward 13 α -hydroxy taxoids or toward 14 β -hydroxy taxoids occur very early following 5 α - and 10 β -hydroxylation of the taxane core, and these findings also have implications for the timing of the C5 α -O-acetylation step involved in taxuyunnanine biosynthesis and thought to play a role in oxetane (D-ring) formation on route to Taxol (**1**).

2. Results and discussion

Preliminary studies were carried out with *Taxus media* var. *Hicksii* suspension cultured cells (line Mh00D) under uninduced and inducing conditions (with methyl jasmonate) as described previously, and the taxoid metabolites were profiled by LC-MS analysis as before (Ketchum et al., 1999;Ketchum et al., 2003;Ketchum and Croteau, 2006). These results, based on several analyses of 14-day old cells in culture, were similar to those observed previously (Ketchum and Croteau, 2006). In uninduced cells (see Figure 2c for a typical total ion chromatogram), the 13 α -hydroxy taxoids constituted roughly 60% of the total mix. This fraction was comprised principally of Taxol (**1**) (about 7% of total metabolite mix), cephalomannine, taxol C, 10-

deacetyl baccatin III, baccatin III, and several other baccatin I, III, IV and VI derivatives that, while containing the 13 α -hydroxyl function, are unlikely to reside on the route to Taxol (**1**) because of inappropriate acylation patterns on the taxane ring system. The 14 β -hydroxy taxoids generally constituted about 15% of the total metabolite mix, although occasionally this family of metabolites nearly doubled in concentration at the expense of the 13 α -hydroxy taxoid types. This fraction was comprised principally of taxuyunnanine C (**5**) (2 α ,5 α ,10 β ,14 β -tetraacetoxytaxa-4(20),11(12)-diene) and a group of related 2,5,10,14-tetraols bearing other side chains and acylation patterns (see Figure 2 and legend). The remaining fraction of the metabolite mix (about 15%) was comprised of a group of about a dozen minor taxoids of uncertain structure; the bulk of these appear to be 13 α -hydroxy taxoids, based on LC-MS analysis (Ketchum and Croteau, 2006), but their structures remain unconfirmed by isolation and NMR analysis. In the case of methyl jasmonate-induced cells, the proportion of 13 α -hydroxy taxoids increased (occasionally to 80% of the product mix) while that of the 14 β -hydroxy taxoids correspondingly decreased, with the level of unknown taxoids remaining roughly constant; this alteration in taxoid distribution upon induction with methyl jasmonate has been previously reported (Ketchum et al., 2003). Some quantitative variation (up to several fold in 14 β -hydroxy taxoid content) was observed in these experiments with induced *Taxus* cells, although qualitative variations were minor.

Preliminary feeding studies with these cultured *T. media* cells were carried out with three common precursors in order to compare incorporation rates into Taxol (**1**) with previous studies which utilized intact *T. brevifolia* tissues as the experimental system. In the case of [20-³H₃] taxadiene (**3**), an incorporation rate of 1.1% into radiochemically pure Taxol (**1**) of *T. brevifolia* stem sections was previously reported (Koepp et al., 1995). The incorporation rate of this early precursor into chromatographically pure Taxol (**1**) in 7-day uninduced *T. media* cells in the present case was 2.9% (corrected for loss of one ³H atom from C20 in the process). For the advanced, labeled precursor baccatin III, an incorporation rate of up to 1% into radiochemically pure Taxol (**1**) of *T. brevifolia* twigs and inner bark pieces was previously reported (Floss and Mocek, 1995). The incorporation rate of [13-³H]baccatin III into chromatographically pure Taxol (**1**) of uninduced *T. media* cells (at 7 days) averaged 4%, with incorporation rates exceeding 11% occasionally observed. For the side chain precursor, labeled β -phenylalanine (which is appended to C13 of baccatin III), the incorporation rates into Taxol (**1**) of uninduced *T. media* cells and into *T. brevifolia* inner bark pieces (Floss and Mocek, 1995) were roughly comparable at 2-3%. Thus, overall, precursor incorporation rates into uninduced *Taxus* cultured cells were generally superior to those of organized tissues of *T. brevifolia*. The corresponding feeding experiments were also carried out with methyl jasmonate-induced *T. media* cells in which the biosynthesis of 13 α -hydroxy taxoids is differentially promoted (Ketchum et al., 2003). These studies showed somewhat greater variability resulting largely from differences in uptake of precursor in these "stressed" cells. Subsequent feeding studies were separately conducted with both induced and uninduced *Taxus* cells; however, the results were so similar that they were combined in the final analysis (see Table 1).

The initial feeding study was carried out with taxa-4(20),11(12)-diene (**3**), the product of the committed pathway step and the presumptive parent of all taxoids (Koepp et al., 1995). This [20-³H₃]-labeled precursor was incorporated into all taxoids in proportional quantities reflecting their approximate relative abundances in the cell extract (Figure 2). For the purpose of comparison, radioactivity incorporated into the 13 α -hydroxy taxoids was summed by integration of radio-peaks, as was radioactivity incorporated into the 14 β -hydroxy taxoids (Table 1), the mean of the summations (59% vs. 26%) again reflecting the approximate proportions of these families of metabolites produced by the cultured cells (additional label was also observed in 5 α -hydroxy- and 5 α ,10 β -dihydroxy-taxadiene intermediates). It should be noted that the radio-abundances of newly synthesized taxoid metabolites may not precisely

match those abundances of the accumulated taxoids because the mix produced during initial growth may not reflect exactly the pathways operating during the feeding period.

In the case of $[20-^3\text{H}_2]5\alpha$ -hydroxytaxa-4(20),11(12)-diene (**4**) as precursor (the first oxygenated derivative of the pathway), the incorporation into 13α -hydroxy taxoids was again much greater than was incorporation into 14β -hydroxy taxoids (Table 1) and the distribution of label within the metabolites of each class was also very similar to that observed with taxadiene (**3**) as precursor (see Figure 2). The results of these first two feeding experiments indicate that both taxa-4(20),11(12)-diene (**3**) and 5α -hydroxytaxadiene (**4**) are incorporated into all taxoids in approximate proportion to their natural abundances, and thus that these two intermediates reside on the same pathway(s) leading to both the 13α -hydroxy taxoids and the 14β -hydroxy taxoids. These findings are consistent with biochemical evidence that the cyclization to taxadiene (**3**) and the C5-hydroxylation of this parent olefin are the first two common steps of taxoid biosynthesis in yew (Croteau et al., 2006).

When the acetylated monool $[20-^3\text{H}_2]5\alpha$ -acetoxytaxa-4(20),11(12)-diene was employed as precursor with *T. media* cells, overall incorporation was comparable to that of the other precursors but the distribution pattern of radioactivity was markedly different. In this case, the acetylated precursor labeled the 14β -hydroxy taxoids with nearly the efficiency of labeling of the 13α -hydroxy taxoids (Table 1). This observation clearly indicates that acetylation of 5α -hydroxytaxadiene (**4**) preferentially diverts pathway flux away from 13α -hydroxy taxoids and toward 14β -hydroxy taxoids, consistent with previous biochemical studies with the 14β -hydroxylase that demonstrated preference for 5α -acetylated derivatives as substrates (Jennewein et al., 2003). Although the 13α -hydroxy taxoids are still formed from 5α -acetoxytaxadiene, they are produced in substantially reduced amounts from the acetate ester compared to 5α -hydroxytaxadiene (**4**) as precursor. This distribution of products derived from 5α -hydroxytaxadiene (**4**) and its acetate ester suggests that a major bifurcation of the taxoid biosynthetic pathway occurs at the level of 5α -hydroxytaxadiene (**4**), with the free alcohol leading, by subsequent hydroxylation, principally to the 13α -hydroxy taxoids, and acetylation, followed by further hydroxylation, leading more favorably to the 14β -hydroxy taxoids.

In the cases of the three racemic substrates described above, the incorporation rates were roughly comparable (13-18%). It is assumed that the *Taxus* cells metabolize only the natural enantiomer of these racemic $[20-^3\text{H}]$ -labeled precursors prepared by total synthesis. No significant anomalous radio-peaks were observed that might be attributed to diastereomeric taxoids that could result from the 'normal' stereoselective metabolism of the 'unnatural' enantiomer of the precursor; however, the residual precursors were not evaluated to determine actual enantiomer depletion.

To further evaluate the proposed pathway bifurcation, i.e., that premature acetylation at C5 promotes formation of the 14β -hydroxy taxoids at the expense of the 13α -hydroxy taxoids, we tested (+)- $[10\alpha-^3\text{H}]5\alpha,10\beta$ -dihydroxytaxa-4(20),11(12)-diene and the corresponding 5α -O-acetate ester as taxoid precursors in cultured *Taxus* cells. The $5\alpha,10\beta$ -diol was efficiently incorporated, and the distribution of label clearly indicated the preferential formation of 13α -hydroxy taxoids over 14β -hydroxy taxoids (Table 1), a pattern reminiscent of that previously observed with 5α -hydroxytaxadiene (**4**). In the case of the 5α -acetate derivative, with which incorporation was again quite efficient, the distribution of label was essentially the same, with the 13β -hydroxy taxoids being preferentially labeled (Table 1), a pattern again similar to the mixture of 13α -hydroxy and 14β -hydroxy taxoids generated by these cells. These results indicate that both $5\alpha,10\beta$ -dihydroxytaxadiene and its 5α -acetate ester reside on the common pathway to both 13α -hydroxy and 14β -hydroxy taxoids, and that separation of the two pathways must occur after C5-acetylation of the $5\alpha,10\beta$ -diol. It is again worth noting that the previously observed pathway redirection toward 14β -hydroxy taxoids upon the "premature"

acetylation of 5 α -hydroxytaxadiene (**4**) is specific to this branch point of taxoid metabolism and is consistent with the substrate preference of the taxoid 14 β -hydroxylase (Jennewein et al., 2003).

3. Conclusions

The results of these feeding studies with simple taxoid precursors (Table 1) indicate that an initial bifurcation in taxoid metabolism occurs very early in the pathway at the level of 5 α -hydroxytaxadiene (**4**) at which the free alcohol proceeds predominantly to the 13 α -hydroxy taxoids, whereas C5-*O*-acetylation diverts pathway flux more favorably toward the 14 β -hydroxy taxoids (Figure 3). The two pathways are not exclusive, in that both 5 α -hydroxytaxadiene (**4**) and the corresponding acetate ester label both families of taxoids, as do the other precursors, but the relative fluxes differ substantially (Table 1).

The acetylation at C5 α at this early stage, with the attendant diversion toward 14 β -hydroxy taxoids, can be regarded as 'premature' in the context of Taxol (**1**) (D-ring) biosynthesis. Thus, acetylation of the C5 α -hydroxyl, at some stage of the pathway, is considered a prelude to oxetane (D-ring) formation via the sequential conversion of the 5 α -acetoxo-4(20)-ene functional grouping to the corresponding β -epoxide, with the last step most plausibly involving intramolecular migration of the 5 α -acetoxo moiety in the process of oxirane ring expansion (Figure 4) (Guéritte-Voegelein et al., 1987; Floss and Mocek, 1995; Giner and Faraldos, 2003). The question therefore arises as to when the acetylation at C5 α occurs on the pathway to Taxol (**1**) and its congeners. The present results suggest that acetylation at C5 α on route to Taxol (**1**) can occur early at the level of 5 α -hydroxytaxadiene (**4**) or 5 α ,10 β -dihydroxytaxadiene because the C5-acetate esters of both give rise to Taxol (**1**), albeit in different proportions with the 5 α ,10 β -diol route preferred (Table 1). In this context, it is important to note that the two 5 α -*O*-acetyltransferases thus far obtained from *Taxus* are both capable of efficiently acylating the C5 α -*O*-position of 5 α -hydroxytaxadiene (**4**) as well as several derivatives bearing additional hydroxyl functions at the 2 α -, 9 α -, 10 β - and 13 α -positions (Chau et al., 2004), thereby supporting the possibility that acetylation at the critical C5 α -hydroxyl function is promiscuous with regard to substrate, and can occur at the level of the monool, diol and even taxoid polyols.

The second important finding to derive from these feeding studies is that 5 α -acetoxo-10 β -hydroxytaxadiene labels the 13 α - and 14 β -hydroxytaxoids in proportion to their production levels in *Taxus* cell culture (Table 1). This finding indicates that the major pathway bifurcation into these nearly mutually exclusive families of metabolites must occur downstream of this intermediate (i.e., after 5 α - and 10 β -hydroxylation, and C5 α -*O*-acetylation, of the taxadiene core). Biochemical studies with the cytochrome P450 taxoid 13 α - and 14 β -hydroxylases, which are presumed to catalyze the committed steps at this critical bifurcation, suggest that these enzymes operate early in taxoid metabolism but the true substrates for, and precise timing of, these reactions is presently uncertain (Lovy Wheeler et al., 2001; Jennewein et al., 2001; Jennewein et al., 2003). Thus, for example, the 13 α -hydroxylase can utilize 5 α -hydroxytaxadiene (**4**) as substrate to afford the 5 α ,13 α -diol product (Jennewein et al., 2001) but the present results now indicate that 13 α -hydroxylation also can occur productively after 10 β -hydroxylation and C5-*O*-acetylation of 5 α -hydroxytaxadiene (**4**).

It would have been very informative in the present study to compare as precursors 5 α ,13 α -dihydroxytaxadiene and its C5-acetate to 5 α ,10 β -dihydroxytaxadiene and its C5-acetate, both to better define the 13 α -/14 β -hydroxy taxoid branch-point(s), and to determine the relative efficiencies of conversion of these taxadiene derivatives to Taxol (**1**). Present evidence suggests that both the 5 α ,13 α -diol and 5 α ,10 β -diol routes to Taxol (**1**) are viable alternatives and converge later in the pathway to the same polyol intermediate (Croteau et al., 2006). However,

the 5 α ,13 α -diol and ester have been prepared thus far only by biosynthesis in amounts too small to permit adequate evaluation of their metabolic disposition and contribution to overall flux in *Taxus* cells. Efforts are underway to synthesize 5 α ,13 α -dihydroxytaxadiene and the C5-ester, as well as the set of triols and tetraols bearing the C5 α -hydroxyl or ester function and with additional hydroxyls at the 2 α -,9 α -,10 β -,13 α - or 14 β -positions. Feeding studies with these precursors to reveal additional diversionary branches, and to elucidate the sequence of steps and the number of potential routes in the production of the presumptive intermediate 2 α -,5 α -,9 α -,10 β -,13 α -pentaol (and its acylated derivatives on the pathway to Taxol (**1**)) and of the presumptive intermediate 2 α -,5 α -,10 β -,14 β -tetraol (and its acylated derivatives on the pathway to taxuyunnanin C (**5**)), will be reported in due course.

4. Experimental

4.1. Substrates and cell cultures

The preparations of ($\pm$)-[20-³H₃]taxa-4(20),11(12)-diene (**3**) (16 Ci/mol), ($\pm$)-[20-³H₂]5 α -hydroxytaxa-4(20),11(12)-diene (**4**) (6 Ci/mol), ($\pm$)-[20-³H₂]5 α -acetyltaxa-4(20),11(12)-diene (6 Ci/mol), and (+)-[10-³H]5 α ,10 β -dihydroxytaxa-4(20),11(12)-diene and the corresponding C5-acetate (both at 11 Ci/mol) have been previously described (Walker et al., 1999; Rubenstein et al., 2000; Lovy Wheeler et al., 2001; Horiguchi et al., 2002). (+)-[13-³H] Baccatin III (9.5 Ci/mol) and ($\pm$)-[1-¹⁴C] β -phenylalanine (5 Ci/mol) were prepared by modification of published methods, as described elsewhere (Walker et al., 2002).

The origin and culturing of *Taxus* suspension cells (including cell lines derived from either *T. media* or *T. cuspidata*) have been described previously (Ketchum and Croteau, 2006), as have the LC-MS based procedures for metabolite profiling of these cells (Ketchum et al., 1999; Ketchum et al., 2003).

4.2 Feeding studies

The appropriate amount of radio-labeled substrate (in ethanol) was added to each well of a sterile 6-well plastic plate (Falcon #353046), and the solvent was removed by evaporation under N₂. An aliquot of *Taxus* suspension cells (that had been growing for 1 week under previously described conditions (Ketchum et al., 2003)), and consisting of 1 ml packed cell volume plus 4 ml media, was then transferred to each sterile well. The plate was then loosely covered, placed in a large plastic bag in an incubator (120 rpm/min, 23°C, in the dark), and maintained for 7 days with periodic venting of the plate (twice daily) to avoid O₂ depletion and CO₂ accumulation. The amounts and concentrations of each substrate for preliminary studies were: taxa-4(20),11(12)-diene (**3**), 10 μ Ci, 125 μ M; baccatin III, 6 μ Ci; 126 μ M; and β -phenylalanine, 4 μ Ci, 160 μ M. For subsequent feeding studies, the amounts and concentrations varied, depending on the experiment, but were typically: taxa-4(20),11(12)-diene (**3**), 2 to 5 μ Ci, 25 to 62.5 μ M; 5 α -hydroxytaxa-4(20),11(12)-diene (**4**) (and acetate), 2 to 5 μ Ci, 67 to 165 μ M; and 5 α ,10 β -dihydroxytaxa-4(20),11(12)-diene (and C5 acetate), 2 to 5 μ Ci, 35 to 87 μ M.

4.3. Product analysis

Following incubation, the contents of each well were transferred to a glass Ten-Broeck homogenizer and homogenized with 3 $\times$ 5 ml portions of CH₂Cl₂. The combined organic extract (~15 ml) was dried over anhydrous MgSO₄, centrifuged briefly to remove solids, and the supernatant was transferred to a glass test tube and evaporated to dryness under N₂. The resulting residue was dissolved in 100 μ l acetonitrile with the aid of sonication, centrifuged briefly to pellet insolubles, and the supernatant filtered through a 0.2 μ m nylon filter in preparation for aliquot counting (by liquid scintillation spectrometry) and metabolite analysis by HPLC.

The analytical system consisted of an Agilent Series 1100 HPLC equipped with diode array detector (monitoring at A₂₁₀, response uncorrected for differing extinction coefficients of the various taxoids), Packard Series A-100 radioactivity detector (using external calibration with each radiolabeled substrate), and Agilent G1946A mass detector (see below) employing Chemstation Software Rev. 8.03. Samples were separated on a Discovery HS-F5 column (250 × 4.6 mm, 5 μm particle size, Supelco) with Metachem Metaguard column using a H₂O:CH₃CN gradient from 5% to 100% CH₃CN at 1 ml/min over 50 min followed by 5 min hold at 100% CH₃CN (Ketchum et al., 2003; Ketchum and Croteau, 2006).

Mass detection of taxoids was by atmospheric pressure chemical ionization (APCI) in the positive ion mode. Drying gas was N₂ at 60 psi, 5 l/min, 350°C. The vaporizer was set to 400°C, fragmentor to 60V, capillary to 3000V, and corona current to 8 μA. Identification of taxoids was accomplished by comparison of retention times and mass fragmentation patterns with authentic standards, and based on previous metabolite profiling of these cells (Ketchum et al., 2003; Ketchum and Croteau, 2006); integrated responses were tabulated then summed for the various taxoid types.

Radioactivity detection of labeled taxoids by flow-through scintillation counting involved valley-to-valley baseline integration of all products eluting in the taxoid region (18 to 46 min; see Figure 2). The 13α-hydroxy taxoids elute from 18 to 32.5 min, whereas the 14β-hydroxy taxoids elute from 33 to 39.5 min. Residual labeled precursors and intermediates that were neither 13α- nor 14β-hydroxy taxoids (i.e., 5α-hydroxy- and 5α,10β-dihydroxy-derivatives of taxadiene) were excluded from the compilations. A total of 129 independent experiments were conducted. Because the distribution of radioactivity from each precursor into 13α-hydroxy- and 14β-hydroxy-taxoids was not significantly influenced by elicitation, cell line, or age of culture when fed, the results for each precursor were combined in the final analysis. Thus, the distribution of radioactivity into 13α-hydroxy taxoids and into 14β-hydroxy taxoids was averaged for each labeled precursor, and the significance of the difference between the means was tested by the paired Student t-test using Minitab software, release 14.2.

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References

- Baloglu E, Kingston DGI. The taxane diterpenoids. *J. Nat. Prod* 1999;62:1448–1472. [PubMed: 10543916]
- Chau M, Walker K, Long R, Croteau R. Regioselectivity of taxoid-*O*-acetyltransferases: heterologous expression and characterization of a new taxadien-5α-*O*-acetyltransferase. *Arch. Biochem. Biophys* 2004;430:237–246. [PubMed: 15369823]
- Croteau R, Ketchum REB, Long RM, Kaspera R, Wildung MR. Taxol biosynthesis and molecular genetics. *Phytochem. Rev* 2006;5:75–97.
- Floss, HG.; Mocek, U. Biosynthesis of taxol. In: Suffness, M., editor. *Taxol - Science and Applications*. CRC Press; Boca Raton, FL, USA: 1995. p. 191-208.
- Giner J-L, Faraldos JA. Facile orthoester formation in a model compound of the taxol oxetane: are biologically active epoxy esters, orthoesters and oxetanyl esters latent electrophiles? *Helv. Chim. Acta* 2003;86:3613–3622.
- Guéritte-Voegelein F, Guénard D, Potier P. Taxol and derivatives: a biogenetic hypothesis. *J. Nat. Prod* 1987;50:9–18. [PubMed: 2885400]

- Hefner J, Rubenstein SM, Ketchum REB, Gibson DM, Williams RM, Croteau R. Cytochrome P450-catalyzed hydroxylation of taxa-4(20),11(12)-diene to taxa-4(20),11(12)-dien-5 α -ol: the first oxygenation step in Taxol biosynthesis. *Chem. Biol* 1996;3:479–489. [PubMed: 8807878]
- Horiguchi T, Rithner CD, Croteau R, Williams RM. Studies on Taxol biosynthesis. Preparation of taxa-4(20),11(12)-dien-5 α -acetoxy-10 β -ol by deoxygenation of a taxadiene tetraacetate obtained from Japanese yew. *J. Org. Chem* 2002;67:4901–4903. [PubMed: 12098303]
- Itokawa, H. Taxoids occurring in the genus *Taxus*. In: Itokawa, H.; Lee, K-H., editors. *Taxus - The Genus Taxus*. Taylor & Francis; London, UK: 2003. p. 35-78.
- Jennewein S, Long RM, Williams RM, Croteau R. Cytochrome P450 taxadiene 5 α -hydroxylase, a mechanistically unusual monooxygenase catalyzing the first oxygenation step of Taxol biosynthesis. *Chem. Biol* 2004;11:379–387. [PubMed: 15123267]
- Jennewein S, Rithner CD, Williams RM, Croteau R. Taxol biosynthesis: taxane 13 α -hydroxylase is a cytochrome P450-dependent monooxygenase. *Proc. Natl. Acad. Sci. USA* 2001;98:13595–13600. [PubMed: 11707604]
- Jennewein S, Rithner CD, Williams RM, Croteau R. Taxoid metabolism: taxoid 14 β -hydroxylase is a cytochrome P450-dependent monooxygenase. *Arch. Biochem. Biophys* 2003;413:262–270. [PubMed: 12729625]
- Ketchum, REB.; Croteau, RB. The taxoid metabolome and the elucidation of the paclitaxel biosynthetic pathway in cell suspension cultures of *Taxus*. In: Saito, K.; Dixon, R.; Willmetzer, L., editors. *Biotechnology in Agriculture and Forestry*. 57. Springer; Heidelberg: 2006. p. 291-309.
- Ketchum REB, Gibson DM, Croteau RB, Shuler ML. The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol. Bioengin* 1999;62:97–105.
- Ketchum REB, Rithner CD, Qiu D, Kim YS, Williams RM, Croteau RB. *Taxus* metabolomics: methyl jasmonate preferentially induces production of taxoids oxygenated at C-13 in *Taxus media* cell cultures. *Phytochemistry* 2003;62:901–909. [PubMed: 12590117]
- Kikuchi, Y.; Yatagai, M. The commercial cultivation of *Taxus* species and production of taxoids. In: Itokawa, H.; Lee, K-H., editors. *Taxus - The Genus Taxus*. Taylor & Francis; London, UK: 2003. p. 151-178.
- Koepp AE, Hezari M, Zajicek J, Stofer Vogel B, LaFever RE, Lewis NG, Croteau R. Cyclization of geranylgeranyl diphosphate to taxa-4(20),11(12)-diene is the committed step of Taxol biosynthesis in Pacific yew. *J. Biol. Chem* 1995;270:8686–8690. [PubMed: 7721772]
- Lovy Wheeler A, Long RM, Ketchum REB, Rithner CD, Williams RM, Croteau R. Taxol biosynthesis: differential transformation of taxadien-5 α -ol and its acetate ester by cytochrome P450 hydroxylases from *Taxus* suspension cells. *Arch. Biochem. Biophys* 2001;390:265–278. [PubMed: 11396929]
- Odgen L. *Taxus* (yews) - a highly toxic plant. *Vet. Hum. Toxicol* 1988;30:563–564. [PubMed: 3245122]
- Rubenstein SM, Vazquez A, Sanz-Cervera JF, Williams RM. Synthesis of stable and radioisotopomers of taxa-4(5),11(12)-diene, taxa-4(20),11(12)-diene and taxa-4(20),11(12)-dien-5 α -ol, early intermediates in taxol biosynthesis. *J. Labelled Compds. Radiopharm* 2000;43:481–491.
- Schoendorf A, Rithner CD, Williams RM, Croteau R. Molecular cloning of a cytochrome P450 taxane 10 β -hydroxylase cDNA from *Taxus* and functional expression in yeast. *Proc. Natl. Acad. Sci. USA* 2001;98:1501–1506. [PubMed: 11171980]
- Takeya, K. Plant tissue culture of taxoids. In: Itokawa, H.; Lee, K-H., editors. *Taxus - The Genus Taxus*. Taylor & Francis; London, UK: 2003. p. 134-150.
- Walker K, Fujisaki S, Long R, Croteau R. Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in Taxol biosynthesis. *Proc. Natl. Acad. Sci. USA* 2002;99:12715–12720. [PubMed: 12232048]
- Walker K, Ketchum REB, Hezari M, Gatfield D, Goleniowski M, Barthol A, Croteau R. Partial purification and characterization of acetyl coenzyme A:taxa-4(20),11(12)-dien-5 α -ol-*O*-acetyl transferase that catalyzes the first acylation step of Taxol biosynthesis. *Arch. Biochem. Biophys* 1999;364:273–279. [PubMed: 10190984]

Fig 1.

Overview of early pathway steps leading to Taxol (**1**) (a typical 13 α -hydroxy taxoid) and taxuyunnanine C (**5**) (a typical 14 β -hydroxy taxoid). Taxoids bearing oxygen functions at both the C13 and C14 positions are quite rare. TS is taxadiene synthase; T5OH is taxadiene 5 α -hydroxylase.

Fig 2.

Identification of taxoid products in extracts of *T. media* cells that had been fed ($\pm$)-[20- 3 H] taxa-4(20),11(12)-diene (**3**). (a) Absorbance at 210 nm (note that taxoids bearing benzoyl groups absorb much more strongly than those lacking benzoyl groups); (b) Radiochromatogram of the taxoid region; (c) Total ion chromatogram (APCI) of the extract. The numbered peaks were identified as:

R_t (min)	Taxoid
20.0	10-Deacetylbaccatin III
22.3	9-Dihydrobaccatin III

R _t (min)	Taxoid
23.6	1 β -Hydroxy-5 α -deacetylbaaccatin I
26.2	9-Dihydro-13-acetylbaaccatin III
27.2	Baaccatin IV
29.2	Taxol D
30.3	Cephalomannine
31.1	Taxol (1)
32.0	Taxol C
32.4	Baaccatin I
33.4	2 α ,5 α ,10 β -Triacetox-14 β -(3-hydroxy-2-methylbutyryloxy)taxa-4(20),11(12)-diene
35.4	2 α ,5 α ,10 β ,14 β -Tetraacetoxytaxa-4(20),11(12)-diene (Taxuyunnanine C (5))
39.2	2 α ,5 α ,10 β -Triacetox-14 β -(2-methylbutyryloxy)taxa-4(20),11(12)-diene
40.8	Unknown non-taxoid
43.1	Unknown non-taxoid
45.3	Taxa-4(20),11(12)-diene (3)

Fig 3.

Scheme for pathway branching at 5α-hydroxytaxa-4(20),11(12)-diene (**4**) by 5-*O*-acetylation and 14β-hydroxylation leading to taxuyunnanine C (**5**) and its congeners, and by 10β-hydroxylation and 5-*O*-acetylation leading primarily to Taxol (**1**) and its congeners. The reverse order (i.e., 5-*O*-acetylation followed by 10β-hydroxylation) also leads predominately to Taxol (**1**) and its congeners. 13α-Hydroxylation followed by 5-*O*-acetylation is considered to represent an alternate pathway leading more or less directly to Taxol (**1**) and related taxoids but the contribution of this route to overall flux is presently uncertain.

Fig 4. Scheme for oxetane (D-ring) formation involving β -epoxidation at C4,C20, and ring expansion with migration of the 5 α -acetoxy group (after Guéritte-Vogelein et al., 1987).

Table 1
Incorporation of early precursors into 13 α -hydroxy taxoids and 14 β -hydroxy taxoids of *Taxus* cells

Precursor	N ¹	Incorporation ² (% of substrate)	13 α -Hydroxy Taxoids (% distribution)	14 β -Hydroxy taxoids	P-value ³
Taxa-4(5),11(12)-diene (3)	28	18	59	26	0.000
5 α -Hydroxytaxa-4(20),11(12)-diene (4)	46	14	73	18	0.000
5 α -Acetoxytaxa-4(20),11(12)-diene	13	13	47	33	0.096
5 α ,10 β -Dihydroxytaxa-4(20),11(12)-diene	34	20	75	14	0.000
5 α -Acetoxy-10 β -hydroxytaxa-4(20),11(12)-diene	8	22	75	18	0.000

¹Total number of experiments (includes all cell lines and treatments).

²Percentage incorporation of labeled precursor into total taxoids (average of two replicates for each substrate from three typical experiments).

³Percentage distribution of labeled precursor into major taxoid types was analyzed by paired Student t-tests (*P*-values indicate the probability that the substrate was equally distributed between the 13 α - and 14 β - hydroxy taxoids).