Taxol Biosynthesis: An Update

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Abstract: The novel diterpenoid taxol (paclitaxel) is now well-established as a potent chemotherapeutic agent. Total synthesis of the drug is not commercially feasible and, in the foreseeable future, the supply of taxol and its synthetically useful progenitors must rely on biological methods of production. The first three steps of taxol biosynthesis have been defined and the responsible enzymes described. These are the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene, the cytochrome P450-catalyzed hydroxylation of this olefin to taxa-4(20),11(12)-dien-5α-ol, and the acetyl CoA-dependent conversion of the alcohol to the corresponding acetate ester. Demonstration of these early steps of taxol biosynthesis suggests that the complete pathway can be defined by a systematic, stepwise approach at the cell-free enzyme level. When combined with in vivo studies to determine contribution to pathway flux, slow steps can be targeted for gene isolation and subsequent overexpression in Taxus to improve the yield of taxol and related compounds.

Key words: Taxol, paclitaxel, biosynthesis, taxadiene synthase, taxadiene hydroxylase, taxadienol acetyltransferase.

Introduction

Very limited supply and early difficulties with formulation did not prevent the development of the diterpenoid natural product taxol* as one of the most useful anticancer drugs now available (1, 2). Taxol has proven effective in the treatment of ovarian and breast cancer, and shows promise for a variety of other cancers such as head and neck, lung, gastrointestinal, and bladder (2, 3). Unlike the Vinca alkaloids, colchicine, and other antimitotic agents that act by preventing the polymerization of tubulin into microtubules, taxol promotes microtubule assembly and suppresses depolymerization (5, 6).

Originally, taxol was obtained by extraction from the peeled bark of the Pacific yew (Taxus brevifolia Nutt.; Taxaceae), a small, slow-growing evergreen tree sparsely dispersed along the coastal and interior mountains of the Pacific Northwest (7). The harvest is destructive and the yields are low; consequently, alternative sources of the drug were sought (8). Nicolaou (9, 10), Holton (11), and others (12) have achieved the total synthesis of taxol by two very elegant routes; however, because of the high costs involved, this approach is not commercially feasible. Currently, taxol and its analog taxotere are being produced semi-synthetically via the acylation of 10-deacetyl-baccatin III (see Fig. 1) and related compounds obtained from the needles of Taxus (13–16). This source of starting material,
although still low yielding, is at least renewable and can be practiced on the plantation scale. *Taxomyces andreanae* and other microorganisms have been reported to produce taxol (17, 18), and several groups have been successful in producing taxol in reasonable titers in *Taxus* cell culture (19, 20) but these production alternatives have not yet proved to be commercially viable. Given the projected increase in taxol usage and the reliance on biological methods of production (8), it is important to understand the biosynthesis of taxol, especially the rate-limiting steps of the pathway which may be manipulated to improve drug yields. This review summarizes recent developments in the study of taxol biosynthesis.

**Previous Work on Taxol Biogenesis**

The structure of taxol is quite complex in bearing an unusual diterpene carbon skeleton, eight oxo-functional groups, and an assortment of appended side-chains, for a total of eleven stereocenters (21) (Fig. 1). Most proposals for taxol formation posit the initial generation of an olefin with the taxane skeleton [as proposed first by Lythgoe (22)], followed by a series of oxygenation steps and, ultimately, acylation reactions (23, 24). Based on precedent in the metabolism of other terpenoid natural products (25), the pathway to taxol from the universal diterpenoid precursor geranylgeranyl diphosphate must involve over a dozen individual enzymatic steps. Given this complexity, the low yields and the operational limitations of the biological sources, deciphering the biogenetic origin of taxol represents a considerable challenge.

Early studies on the biogenesis of taxol involved in vivo feeding experiments with radiolabeled precursors that seemed to indicate that acetate, mevalonate, and phenylalanine represented the building blocks of taxol (26). Recently, however, Zenk and his associates (27) have shown conclusively that mevalonate is not a precursor of the diterpenoid taxane ring system but rather have implicated the origin of the key intermediate isopentenyl diphosphate by an alternate pathway for isoprenoid biosynthesis proposed by Rohmer (28). In exploring later stages of the taxol pathway, Floss and his colleagues have carried out detailed feeding studies with advanced metabolites to demonstrate the origin and timing of assembly of the N-carbons (29, 30). A description of this work and an excellent overview of previous speculations on the biogenetic pathway(s) leading to taxol have been provided by Floss and Moczek (24).

**First Step in Taxol Biosynthesis: Taxadiene Formation**

The committed step in the biosynthesis of taxol and related taxoids is the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (Fig. 1) (31), not the exocyclic isomer, taxa-4(20),11(12)-diene, as earlier proposed based on the abundance of taxane metabolites with this structure (23, 24). In vivo experiments with *T. brevifolia* stem sections, using labeled taxa-4(5),11(12)-diene, demonstrated the very efficient incorporation (30%) of the olefin into taxol and closely related taxoids, and the endocyclic olefin was demonstrated to be present in *T. brevifolia* bark at low levels (5–10 µg per kg dry wt.), whereas the exocyclic isomer was not detected (31). Both taxa-4(5),11(12)-diene and the 4(20)-isomer were subsequently synthesized by Rubenstein and Williams (32).

A cell-free system from *T. brevifolia* sampling stem was shown to catalyze the divergent metal ion dependent conversion of geranylgeranyl diphosphate to taxadiene as essentially the only olefinic product (33). This operationally soluble enzyme was localized mainly in the bark and adhering cambium cells, and a 600-fold conventional purification, by chromatographic and electrophoretic means, yielded the pure protein which the combination of gel permeation chromatography and SDS-PAGE showed to be a monomer of ~79 kDa (33). This taxadiene synthase has been characterized with regard to pH optimum, kinetic constants, and possible active site residues, and shown to prefer Mg²⁺ (Kₘ ~ 0.16 mM) to Mn²⁺ and other divalent metal ions as the required cofactor.

The cyclization reaction has been examined in some detail and shown to proceed without detectable free intermediates or the preliminary formation of enzyme-bound taxa-4(20),11(12)-diene with subsequent isomerization, and to involve an intramolecular hydrogen transfer from C-11 of a verticillene intermediate to the C-ring of taxa-4(5),11(12)-diene (34). From these results, a stereochemical mechanism has been proposed for the taxadiene synthase reaction involving the initial cyclization of geranylgeranyl diphosphate to a transient verticillene cation intermediate, with transfer of the C-11 α-proton to C-7 to initiate transannular B/C-ring closure to the taxenyl cation, followed by deprotonation at C-5 to yield the taxa-4(5),11(12)-diene product directly (Fig. 2).

The low levels of taxadiene synthase activity in *Taxus* stem tissue (140 pmol·h⁻¹·g⁻¹), the very low abundance of taxadiene in bark, and the efficient conversion of the olefin to...
taxoids in vivo suggested that the cyclization of geranylgeranyl diphosphate to taxadiene was slow relative to downstream steps in the biosynthetic pathway to taxol. The potential role of this first committed step in the control of pathway flux prompted a search for the cDNA encoding taxadiene synthase. Attempts to obtain amino acid sequence information from the enzyme, for use as a cloning tool, were unsuccessful, and led to the adoption of a PCR-based strategy built upon homologous sequences of other known terpenoid cyclases of plant origin. One set of primers, of the many tested, yielded a small DNA fragment that was “cyclase-like” in sequence and that was subsequently employed as a hybridization probe to screen a cDNA library from T. brevifolia stem. Several of the cDNA clones so isolated were full-length and were functionally expressed in Escherichia coli, thereby providing confirmation by the conversion of geranylgeranyl diphosphate to taxadiene (35). The sequence specifies an open reading frame of 2586 nucleotides, and the complete deduced polypeptide contains 862 amino acid residues, including a long plastidial targeting sequence, corresponding to a molecular weight of about 98,000. Sequence comparison with other terpenoid synthases from higher plants showed a significant degree of similarity (especially with those of other gymnosperms), supporting the suggestion of a common ancestry for this class of enzymes (35).

Since an assessment of the possible role of taxadiene synthase in the control of pathway flux to taxol could not be easily carried out in intact Taxus stem tissue, heterotrophic suspension cell cultures of the Canadian yew (Taxus canadensis) were employed as an experimental system. The enzyme from this source was isolated and shown to be chromatographically, electrophoretically, and kinetically identical to the taxadiene synthase of T. brevifolia stem (20). Results from the analysis of enzyme activity levels during the time-course of taxol accumulation in developing cell cultures indicated a rise in taxadiene synthase activity before taxol accumulation began, which persisted into stationary phase, and also that synthase activity in vitro exceed the maximum rate of taxol accumulation in vivo (20). Combined, these results suggest that, although taxadiene synthase represents a slow biochemical transformation and, thus, a worthy candidate for gene isolation.

Second Step in Taxol Biosynthesis: Taxadienol Formation

No oxygenated taxoids bearing a 4(5)-double bond are known, whereas taxoids with the 4(20)-ene-5-oxy functional grouping are exceedingly common (36), suggesting that hydroxylation of taxa-4(5),11(12)-dien-5a-ol. Membranous fractions were inactive in the acetylation reaction. The soluble enzyme preparation from T. canadensis suspension cultured cells was shown to catalyze the acetylation of taxa-4(20),11(12)-dien-5a-ol could be the next step in taxol biosynthesis. Additionally, the 4(20)-ene-5a-acetate functional group could play a role in the elaboration of the oxetane ring of taxol by a mechanism involving epoxidation of the 4(20)-double bond followed by intramolecular acetate migration with oxirane ring expansion (Fig. 3) (23, 24, 39). These considerations, as well as studies on subsequent oxygenation steps (see below), prompted an evaluation of the enzymatic acylation of taxa-4(20),11(12)-dien-5a-ol.

A soluble transacetylase has been partially purified and characterized as a 50 kDa monomeric protein (with $pI \sim 4.7$) that accepts acetyl CoA, but not benzoyl CoA, as a cosubstrate.

![Fig. 3](https://example.com/fig3.png) A possible mechanism for the construction of the oxetane ring of taxol from the 4(20)-ene-5a-acetoxy functional grouping. Epoxidation of the 4(20)-double bond, followed by intramolecular acetate migration and oxirane ring opening, could furnish the oxetane moiety.
Subsequent Oxygenation Steps

Subsequent reactions on the taxol pathway include additional oxygenations, hydroxy group acylations, oxidation to a ketone, and generation of the oxetane ring system. The large number of naturally occurring taxoid metabolites with a broad range of oxygenation and acylation patterns provides little guidance in predicting the order of functionalization beyond taxa-4(20),11(12)-dien-5α-yl acetate. Consideration of the relative abundance of taxoids bearing an oxygen function at each carbon of the taxane ring has suggested the order of oxygenation to be C-5 followed by C-10, then C-2 and C-9 (24, 41). Metabolites bearing an oxygen at C-13 are next in abundance, thus suggesting a sequence for the conversion of taxadiene to the level of a pentaol (Fig. 4). Oxygenations at C-7 and C-1 of the taxane nucleus are considered to be late introductions, possibly occurring after oxetane ring formation. However, oxetane formation, itself a relatively late-stage transformation, seemingly must precede acylation at C-13 and oxidation of the C-9 hydroxy to a carbonyl (cf. Fig. 1) (24, 41).

![Taxa-4(5),11(12)-diene](image)

![Taxa-4(20),11(12)-dien-5α-ol](image)

Microsomal preparations of T. canadensis cell cultures, when incubated with taxa-4(20),11(12)-dien-5α-ol in the presence of NADPH and O2, yield an enzymatic product more polar than the substrate, with chromatographic properties and mass spectrum (by HPLC-MS) consistent with a taxadiene diol. The reaction, like the hydroxylation of taxadiene, is catalyzed by a cytochrome P450 hydroxylase, suggesting that the remaining oxygenation steps in taxol biosynthesis may involve similar enzymes. By utilizing the microsomal system that had been carefully optimized to sustain cytochrome P450-type reactions (38), both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-dien-5α-ol as substrates gave rise to products that were tentatively identified by HPLC-MS analysis as the corresponding diol, triol, and tetraol; the precise structures of these products are as yet uncertain. Incubation of the optimized microsomes with taxa-4(20),11(12)-dien-5α-yl acetate as substrate yielded a single major product that, based on HPLC-MS evaluation, appeared to be a pentaol monooacetate. Taxa-4(20),11(12)-dien-5α-yl benzote was not detectably oxygenated by this system, indicating that the efficiency of the acetate ester as precursor was not due simply to increased lipophilicity but rather to improved recognition of this substrate by this set of microsomal hydroxylases that appear to operate sequentially. This successive series of hydroxylations is now being systematically characterized with regard to substrates and the structural elucidation of the corresponding products, and with consideration to the possibility of intervention of additional acylation steps in the sequence.

Conclusion

Improvement of the biological production of taxol, and of related taxoid metabolites of use in drug semi-syntheses (42), should be based on a thorough understanding of the complex target pathway and its rate limiting steps. A systematic, stepwise approach should reveal the sequence of oxygenation and acylation steps, the timing of the dehydrogenation at the C-9 hydroxy and the formation of the unique oxetane ring, and the coordination of these processes with the assembly of the N-benzoyl phenylisoserine side chain (29, 30). The role of each enzymatic step in the control of pathway flux can then be assessed by in vivo studies, the slow steps revealed, and suitable cloning strategies devised to acquire the corresponding genes. This approach will ultimately lead to engineering of Taxus or relevant microorganisms that overexpress key genes to increase flux through the corresponding enzyme catalyzed steps in order to improve production yields of taxol.

![Fig. 4 Proposed sequence for the hydroxylation of taxa-4(5),11(12)-diene to the level of a pentaol based on the relative abundances of naturally occurring taxoids.](image)

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