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Regioselectivity of taxoid-*O*-acetyltransferases: heterologous expression and characterization of a new taxadien-5α-ol-*O*-acetyltransferase☆,☆☆

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Abstract

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

Keywords: Taxol; Paclitaxel; *Taxus*; Taxane diterpenoid; Acetyltransferase; Taxadien-5α-ol-*O*-acetyltransferase; Taxadien-5α-ol; Taxadien-5α-yl acetate; Taxadien polyols

Taxoids (taxane diterpenoids), isolated from yew (*Taxus*) species, consist of a group of highly functionalized diterpenes, most of which possess the unique pentamethyl $[9.3.1.0]^{3,8}$ tricyclopentadecane (taxane) skeleton like that found in the anticancer drug Taxol [1]. Although Taxol and its precursors for semisynthesis are arguably the most important taxoids, an examination of a compendium of the naturally occurring taxoids [2] reveals a large assortment of other compounds possessing positionally different acylation patterns and a variety of acyl and aroyl

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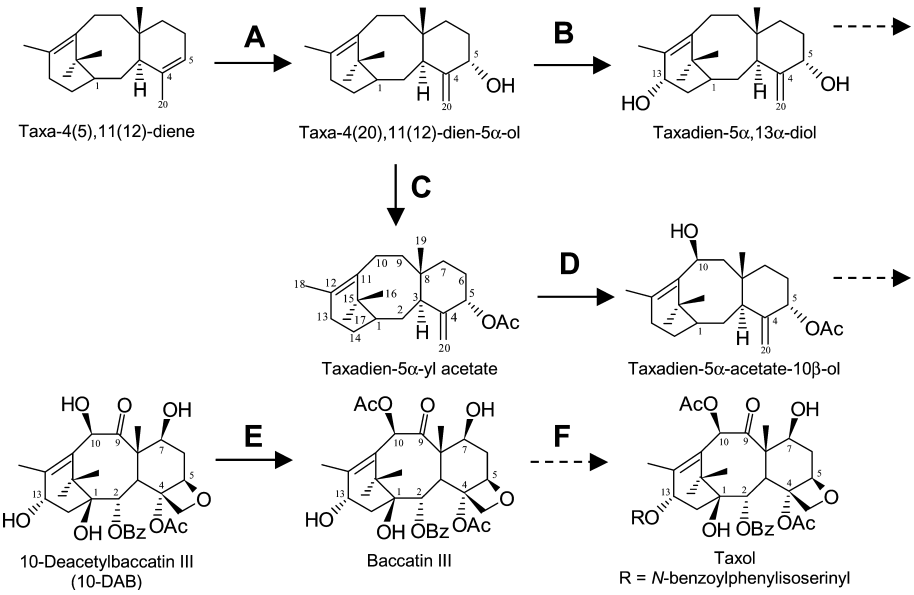


Fig. 1. Outline of the Taxol biosynthetic pathway. Hydroxylation of the committed precursor taxa-4(5),11(12)-diene to taxadien-5α-ol by the taxoid 5α-hydroxylase (A), hydroxylation of taxadien-5α-ol by the taxoid 13α-hydroxylase (B), acetylation of taxadien-5α-ol by the taxadienol-*O*-acetyltransferase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

substitutions. These latter functional groups include acetyl, propionyl, and butyroyl esters, and their hydroxylated and methylated derivatives, tigloyl and benzoyl esters, and amino acid derivatives, such as cinnamoyl, aminophenylpropanoyl (Winterstein's acid), and phenylisoserinoyl esters, as well as occasional glycosyl groups. Taxol (Fig. 1, generic name paclitaxel) bears acetate groups at C4 and C10, a benzoate at C2, and the unusual *N*-benzoylphenylisoserine side chain appended at C13 (the C2-benzoate, C13-side chain, and C4-acetate are important in the pharmacophore for promoting tubulin binding that underlies the cytotoxic mode of action of Taxol [3]). However, taxoids bearing acetate groups variously at C1, C2, C5, C7, C9, C10, and C13 positions are far more numerous than Taxol and its congeners.

The rationale for the production of such a vast assortment of acetylated taxoids is unknown. Some of these metabolites may be relevant intermediates, or may simply represent the consequence of promiscuous acyltransferase activity, while others may play a role in plant defense in possessing antifeedant [4] and antifungal [5,6] activities, and toxicity towards mammals [7,8]. What is clear is that *Taxus*, both intact plants and derived cell cultures [9], direct considerable pathway flux to the production of acetylated taxoids rather than Taxol, and that any approach to improving the production yields of Taxol and its immediate precursors must take into account these apparently diversionary taxoid biosynthetic side-routes.

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

Genes encoding the acyl and aroyltransferases of Taxol biosynthesis have been cloned by several strategies employing cDNA libraries derived from transcripts isolated from *Taxus* cell cultures induced with methyl jasmonate for increased Taxol production [11]. These approaches, in addition to the random sequencing of an EST[2] library from similarly induced *Taxus* cells

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

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

[24], have now yielded a family of 16 closely related acyltransferase clones, including the above-described five [18–22] which have been characterized by heterologous functional expression in *Escherichia coli*. The recent acquisition of this family of presumptive taxoid acyl/aroyltransferase clones has provided the opportunity to broaden our search for genes relevant to taxoid metabolism, with immediate focus on those responsible for the origin of acetylated taxoids and with a long-term view toward manipulating these genes so as to suppress side-routes and promote pathway flux towards Taxol. In this paper, we report the cloning and characterization of a new taxoid acetyltransferase (denoted TAX19), and a comparison of the properties of the recombinant enzyme with those of previously isolated taxoid-*O*-acetyltransferases (TAX1 and TAX6), and we discuss the implications of these findings for the biosynthesis of Taxol and related acetylated taxoids.

Materials and methods

Substrates

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

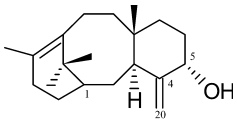
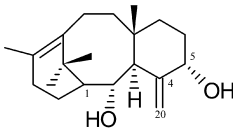
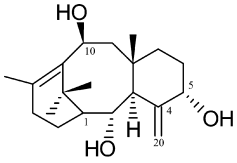
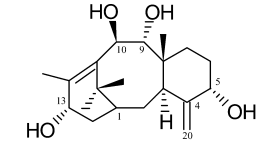
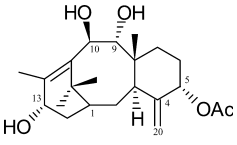
Heterologous expression in E. coli and functional screening

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

Partial purification of recombinant acetyltransferases

Following the above screening, and prior to purification, the soluble enzyme extracts of *E. coli* expressing the target clones were analyzed by SDS–PAGE and Coomassie brilliant blue staining [29] to verify production of the recombinant proteins (~50 kDa) relative to a comparable preparation from the negative control harboring empty vector. *E. coli* cultures confirmed to express the target acyltransferase genes were grown in 1 L LB media (*E. coli* harboring TAX1 was grown in the presence of 1 M sorbitol and 2.5 mM betaine to

Table 1
Major product formed by, and kinetic analyses of, TAX1 and TAX19 acetyltransferases

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

The taxoid test substrates are shown in the left column, along with the corresponding K_M, k_{cat}, and catalytic efficiency values determined with the partially purified enzymes at optimum pH and saturating acetyl CoA concentration.

reduce the formation of inclusion bodies [30]), shaken at 37 °C until cell density increased to A_{600} ~0.5, and then induced with 0.5 mM IPTG and grown for another 24 h at 18 °C. The transformed bacteria were harvested by centrifugation (6000*g* for 20 min), washed with phosphate buffered saline, and centrifuged again. The pelleted cells were resuspended in extraction buffer (50 mM Mopso, pH 7.2, with 5% v/v glycerol, and 0.5 mM DTT) and lysed by sonication (3 × 30 s) on ice using a Virsonic 475 (Virtis, Gardiner, NY) at medium power with the 0.5 in. probe. The derived homogenate was centrifuged (10,000*g*) for 20 min, and the resulting supernatant was centrifuged again at 45,000*g* for 1 h to provide the soluble enzyme extract. Using a Bio-Rad BioLogic LP pump with UV-monitoring at 254 nm, the protein extract (50 mL) was loaded onto a diethylaminoethyl-cellulose (10 g, Whatman DE-52, Clifton, NJ) column that was previously equilibrated with 50 mM Mopso, pH 7.2, containing 5% v/v glycerol, 5 mM $MgCl_2$, and 1 mM DTT (equilibration buffer) and eluted with a linear NaCl gradient (0–100 mM; 200 mL, 2 mL/min) in the same buffer. Fractions containing the target enzyme (eluting between 30 and 65 mM), as determined by the above assay, were pooled and loaded (2 mL/min) onto a ceramic hydroxyapatite (10 g, Bio-Rad, Hercules, CA) column preequilibrated with the above equilibration buffer. The column was washed (3 mL/min) with 25 mL of equilibration buffer, followed by 50 mL of 50 mM Mopso, pH 7.2, containing 1 M NaCl, then again with 25 mL of equilibration buffer, followed by elution with a linear gradient of 0 to 50 mM potassium phosphate buffer (pH 7.2, 200 mL, 3 mL/min). Fractions containing the acetyltransferases free of competing activities (eluting between 10 and 25 mM), as determined by assay, were analyzed by SDS–PAGE with Coomassie brilliant blue staining, and these fractions were pooled, concentrated using an Amicon Centriprep YM-30 centrifugal concentrator, and used for subsequent characterization studies. An aliquot of the pooled concentrated protein (12 μg) was analyzed by SDS–PAGE to

determine the relative percentage of recombinant protein with respect to total protein present for the purpose of calculating k_{cat}. A 1 L culture of *E. coli* harboring TAX19 yielded ~5 mg of partially purified acyltransferase (60% pure); a 1 L culture of *E. coli* harboring TAX1 yielded ~1 mg of partially purified acyltransferase (40% pure). TAX1 preparations were scaled up fivefold for routine use.

Product identification and enzyme characterization

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

Kinetic values for taxadien-5α-ol, taxadien-2α,5α-diol, and taxadien-2α,5α,10β-triol (all at a concentration range of 2–100 μM), taxadien-5α,9α,10β,13α-tetraol (at a concentration range of 5–300 μM), and taxadien-5α-acetoxy-9α,10β,13α-triol (at a concentration range of 5–500 μM) were determined by Eadie–Hofstee plotting using Microsoft Excel software; assays were performed at the optimum pH of 8 in 100 mM phosphate buffer at operational saturation of [^{3}H]acetyl CoA. The reported values are means ± SD of two independent replicates.

For structural elucidation of biosynthetic products, the assays were scaled up sufficiently (in 100 mM phosphate buffer, pH 8.0, with 5 mM DTT and 100 μM each of acetyl CoA and taxoid substrate; 4 h at 31 °C) to generate about 1 mg of material. The products were extracted as before and purified by HPLC on a Metachem 5μ Taxsil column with isocratic elution (2:3, CH_3CN:H_2O). The purified material was concentrated to remove CH_3CN, NaCl was added to the residual aqueous phase, and the product was extracted twice with an equal volume of diethyl ether. The ether was evaporated, and the residual material was lyophilized and then dissolved in 100% $CDCl_3$ for NMR analysis. ^{1}H NMR, NOESY, COSY, and TOCSY experiments were performed on a Varian Mercury 300 MHz instrument. Coupled GC–MS analyses were performed on a Hewlett–Packard 6890 MSD system (electron impact at 70 eV ionizing voltage) using a Phenomenex ZB5 column (30 m × 0.25 mm, 5% phenyl polysiloxane) with cool on-column injection, and programming from 40 °C at 30 °C/min to 220 °C, then 2 °C/min to 260 °C, and finally 20 °C/min to 320 °C.

The taxadien-5α,9α,10β-triacetoxy-13α-ol standard was generated chemically from taxadien-9α,10β-diacetoxy-5α,13α-diol [16] by TES protection of the C13-hydroxyl, followed by acetylation of the C5-hydroxyl [16], and TES deprotection using HF/pyridine [31]. This product was purified using a previously described method [16] (yield ~25%, >95% purity by NMR) and analyzed by ^{1}H NMR: δ; 0.74 (*s*, CH_3), 0.96 (*s*, CH_3), 1.15 (*m*, H14α), 1.49 (*s*, CH_3), 1.72 (*m*, H6), 1.76 (*m*, H2), 1.77 (*m*, H1), 1.88 (*m*, H7), 2.02 (*s*, $COCH_3$), 2.06 (*s*, $COCH_3$), 2.12 (*s*, $COCH_3$), 2.23 (*d*, *allylic*-CH_3, J = 1.2 Hz), 2.84 (*dt*, H14β, J = 3.4, 10.8, 16.2 Hz), 3.05 (*br s*, H3), 4.45 (*dd*, H13, J = 4.5, 11.1 Hz), 4.89 (*d*, H20, J = 2.2 Hz), 5.23 (*d*, H20, J = 1.5 Hz), 5.37 (*t*, H5, J = 3.3 Hz), 5.79 (*d*, H9, J = 10.5 Hz), 6.06 (*d*, H10, J = 10.5 Hz).

Results and discussion

Preliminary screening of acetyltransferase clones

Screening of the *Taxus* acyl/aroyltransferase clones, verified to express the recombinant enzyme by SDS–PAGE, was conducted using soluble protein extracts of the corresponding transformed bacteria with all available taxoid substrates and acetyl CoA as co-substrate, and with product assessment by GC–MS and radio-HPLC. The previously described recombinant 10-deacetylbaccatin III-10-*O*-acetyltransferase (TAX6) [21] was shown to acetylate taxadien-5α,9α,10β,13α-tetraol to the corresponding tetraol-9-acetate, tetraol-10-acetate, and tetraol-9,10-diacetate, and to convert taxadien-5α-acetoxy-9α,10β,13α-triol to the corresponding tetraol-5,9-diacetate and tetraol-5,10-diacetate. None of the other test substrates was measurably functional with this enzyme. These newly described activities of the 10-deacetylbaccatin III-acetyltransferase (see Fig. 1) are consistent with the utilization of moderately to highly functionalized taxoids by this enzyme, with a common "northern hemisphere" regioselectivity. The previously characterized taxoid side chain-*N*-benzoyltransferase (TAX10) [19] and a new EST-derived acyltransferase clone (designated TAX14) were found to selectively convert taxadien-5α,13α-diol [14] to the same diol monoacetate; however, due to limited availability of this substrate, the product could not be prepared in sufficient amount to determine identity. TAX14 was also shown to convert taxadien-5α,10β-diol to the 5-monoacetate derivative. The previously described taxoid 5α-acetyltransferase (TAX1) [22] and a previously undefined

acyltransferase (TAX19) were both found to acetylate a range of taxoid substrates, including taxadien-5α-ol, taxadien-5α,10β-diol, taxadien-5α,13α-diol, taxadien-2α,5α,10β-triol, taxadien-5α,9α,10β,13α-tetraol, and taxadien-5α-acetoxy-9α,10β,13α-triol. The TAX19 enzyme was also able to utilize taxadien-2α,5α-diol as substrate (Table 1). Because of the range of substrates utilized and sequence considerations (see below), the TAX1 and TAX19 acetyltransferases were examined in greater detail. Both recombinant enzymes were fractionated to remove competing hydrolases and provide sufficient purification without undue loss of activity (~50% pure) to allow accurate calculation of k_{cat} values.

Substrate selectivity and regioselectivity of TAX1 and TAX19

At optimum pH and saturating CoA levels, both partially purified TAX1 and TAX19 acyltransferases were able to convert taxadien-5α-ol to taxadien-5α-yl acetate (Table 1); this is the original activity for which TAX1 was designated a taxoid 5α-*O*-acetyltransferase [22]. Like the TAX10 and TAX14 acyltransferases described above, the TAX1 and TAX19 enzymes were also capable of acetylating taxadien-5α,13α-diol to the same diol monoacetate but, unlike TAX10, these other enzymes could additionally acetylate taxadien-5α,10β-diol to taxadien-5α-acetoxy-10β-ol (product identification by HPLC-based comparison to the authentic standard [13]). One interesting difference with respect to substrate utilization was demonstrated by the ability of the TAX19 acetyltransferase to acetylate taxadien-2α,5α-diol; the TAX1 enzyme had no detectable activity with this substrate. The acetylated product of taxadien-2α,5α-diol was analyzed by GC–MS (R_t 17.61 min) and yielded a fragmentation pattern consistent with a diol monoacetate. The parent ion (P^+) was observed at *m/z* 346, as were diagnostic fragment ions at *m/z* 328 (P^+–H_2O), 313 (P^+–H_2O–CH_3), 286 (P^+–CH_3COOH), 268 (P^+–CH_3COOH–H_2O), and 253 (P^+–CH_3COOH–H_2O–CH_3). Subsequent studies with taxadien-2α,5α,10β-triol as substrate revealed the first example of a regiospecific difference between the TAX1 and TAX19 acetyltransferases. The TAX19 enzyme was capable of acetylating this taxoid triol to taxadien-5α-acetoxy-2α,10β-diol (identification by GC–MS comparison to the authentic standard [27]). The regiospecific acetylation at the C5 hydroxyl (but not the C2 hydroxyl) of this 2,5,10-triol suggests that the TAX19 transferase also regiospecifically acetylated the C5 position of the above taxadien-2,5-diol (but this identification was not confirmed). TAX1 transferase, on the other hand, also acetylated this taxadien–triol but the product had a different HPLC retention than did the C5-acetoxy derivative produced by TAX19, indicating that acetylation by TAX1 occurred at either the C2 or C10 positions (likely the C10-hydroxyl; see below).

The TAX1 and TAX19 transferases were also both capable of converting taxadien-5α,9α,10β,13α-tetraol to a number of different acetylated products (Table 2). One product formed by the TAX1 enzyme (25% of product mix; R_t 27 min by HPLC) was purified by HPLC (~1 mg, >95% purity) and the structure was determined by ^{1}H NMR: δ; 0.91 (*s*, CH_3), 1.01 (*s*, CH_3), 1.2 (*dd*, H14α, J = 6.9, 13.8 Hz), 1.43 (*s*, CH_3), 1.53 (*m*, H7), 1.66 (*m*, H2), 1.7 (*m*, H1), 1.74 (*m*, H6), 2.02 (*s*, $COCH_3$), 2.16 (*d*, *allylic*-CH_3, J = 1.2 Hz), 2.87 (*dt*, H14β, J = 3.6, 9.3, 15.6 Hz), 3.00 (*br s*, H3), 4.35 (*t*, H5, J = 3 Hz), 4.43 (*br d*, H13, J = 7.2 Hz), 4.72 (*d*, H20, J = 1.2 Hz), 4.90 (*d*, H10, J = 11.7 Hz), 5.11 (*s*, H20), 5.60 (*d*, H9, J = 12 Hz). Based on these proton assignments, and spectrometric comparison with the substrate tetraol and its peracetylated derivative (taxusin), this product was confirmed as taxadien-9α-acetoxy-5α,10β,13α-triol. A second product (55% of the product mix) formed by the TAX1 transferase with the taxadien–tetraol substrate had a slightly later retention time than the first (R_t 28 min by HPLC), and this product was also purified (~1 mg, >90% purity) and the structure was determined by ^{1}H NMR: δ; 0.89 (*s*, CH_3), 0.91 (*s*, CH_3), 1.24 (*dd*, H14α, J = 4.5, 10.5 Hz), 1.40 (*s*, CH_3), 1.68 (*m*, H2), 1.7 (*m*, H6), 1.7 (*m*, H1), 1.8 (*m*, H7), 2.11 (*s*, $COCH_3$), 2.22 (*d*, *allylic*-CH_3, J = 1.5 Hz), 2.81 (*dt*, H14β, J = 3.6, 8.9, 14.7 Hz), 3.25 (*br s*, H3), 4.12 (*d*, H9, J = 9.9 Hz), 4.34 (*br s*, H5), 4.37 (*br s*, H13), 4.71 (*s*, H20), 5.07 (*s*, H20), 5.89

Table 2
Distribution of products formed by TAX1 and TAX19 acetyltransferases with taxadien-5α,9α,10β,13α-tetraol (tetraol) and taxadien-5α-acetoxy-9α,10β,13α-triol (tetraol-5OAc) as substrates

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

The acetylated hydroxyl group(s) and the relative percentage conversion rates are shown for each substrate.

(*d*, H10, $J = 9.9$ Hz). Based on these assignments, and comparison with reference NMR spectra of the starting tetraol, taxusin, and other tetraol derivatives [16], this product was confirmed as taxadien-10β-acetoxy-5α,9α,13α-triol. Similar methods and comparison to authentic standards [16] were used to determine the identities of the remaining TAX1 and TAX19 enzyme products, and to demonstrate that the major product formed by the TAX19 acetyltransferase from taxadien-5α,9α,10β,13α-tetraol was taxadien-13α-acetoxy-5α,9α,10β-triol (Table 2). With the TAX1 transferase, the major product formed from taxadien-5α-acetoxy-9α,10β,13α-triol was taxadien-5α,9α,10β-triacetoxy-13α-ol, and with the TAX19 transferase, the major product from this substrate was taxadien-5α,13α-diacetoxy-9α,10β-diol (Table 2); these results are entirely consistent with the regioselectivities of the respective enzymes observed with the taxadien–tetraol substrate.

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

Kinetic analyses of TAX1 and TAX19

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

Both TAX1 and TAX19 exhibited the lowest K_M values (and respectable turnover rates) for taxadien-5α-ol, an early intermediate of Taxol biosynthesis [32], suggesting that acetylation at C5 is of relevance in vivo as an early pathway step. Based on utilization of the other test substrates, TAX19 appears to be the more promiscuous of the two transferases but both enzymes do catalyze reactions ''off pathway'' for Taxol production (i.e., C9 acetylation by TAX1 and C13-acetylation by TAX19). Based on the high K_M values for both enzymes in the utilization of taxadien-5α-acetoxy-9α,10β,13α-triol, it might be argued that C5-acetoxy taxadien polyols are not relevant substrates for these enzymes in vivo; however, the k_{cat} values for this substrate were also remarkably high, thus yielding quite respectable catalytic efficiencies (Table 1). The K_M value for acetyl CoA with TAX1 was previously reported as 5.5 μM [33]; the K_M value for acetyl CoA with TAX19 was determined in this study to be 5.2 ± 2.5 μM. Kinetic parameters in the present study were measured by total (multiple) product formation. It is appreciated that these kinetic values represent a mixture of reaction channels and (presently indistinguishable) binding modes for the taxoid substrate and/or acetyl CoA. The kinetic constants for TAX1 and TAX19 compare favorable with those of the 10-deacetylbaccatin III-10-*O*-acetyltransferase (TAX6) with K_M values of 10 and 8 μM for 10-deacetylbaccatin III and acetyl CoA, respectively [21].

Sequence analyses of acetyltransferases

The TAX19 cDNA (orf 1329 bp) encodes a 442-residue protein with a calculated molecular weight of 49,290. This size is consistent with the results of SDS–PAGE analysis of the recombinant protein and is very similar to that of the TAX1 taxadienol-5α-*O*-acetyltransferase previously described [22,33]. The cDNA sequence does not appear to encode protein N-terminal targeting information, consistent with the presumptive cytosolic localization of the enzyme and its operationally soluble nature. Examination of the deduced amino acid sequence (Fig. 3) revealed the highly conserved HXXXDG catalytic triad (aa 164–169) found in other acyltransferases [11,34–36]. Site-directed mutagenesis of the histidine of the triad has shown that it is essential for catalytic activity, and it has been suggested that this residue acts as a general base in the catalytic transfer of the acyl moiety from acyl CoA to the substrate hydroxyl group [34,35]. It has also been reported that a cysteine residue is important for activity of *O*-acetyltransferases [37]. An alignment (GCG, Madison, WI, version 10; gap creation penalty of 3, gap extension penalty of 1) of the defined *Taxus* acyltransferases (Fig. 3) reveals two cysteines, in addition to the one of the HXXCDG motif, that are highly conserved, one at position 96 (of TAX19) and the other which is a part of a CGG motif (aa 153–155). Another conserved element of enzymes of this type is the DFGWG motif (aa 375–379) [35].

Pair-wise comparisons (GeneDoc, version 2.6.002) of TAX1 and TAX19 reveal moderate sequence similarity (*S*) and identity (*I*) scores of 77 and 61%, respectively. Comparisons of the TAX1 and TAX19 sequences with the other described acyl/aroyltransferases of Taxol biosynthesis also showed similar scores (~75% *S*, ~60% *I*). However, one comparison did reveal very high homology; TAX14 and TAX19 exhibit similarity and identity scores of 93 and 87%, respectively. Although these sequences are highly homologous, the corresponding enzymes utilize only two substrates in common (taxadien-5α,10β-diol and taxadien-5α,13α-diol). Interestingly, the TAX1 and TAX19 sequences are not as similar, but the corresponding enzymes utilize several substrates in common with comparable activities. At our present level of understanding, it is not possible to relate primary structure to substrate utilization by this enzyme type. Neither of the latter acetyltransferases is highly abundant in the induced *Taxus* cell EST library

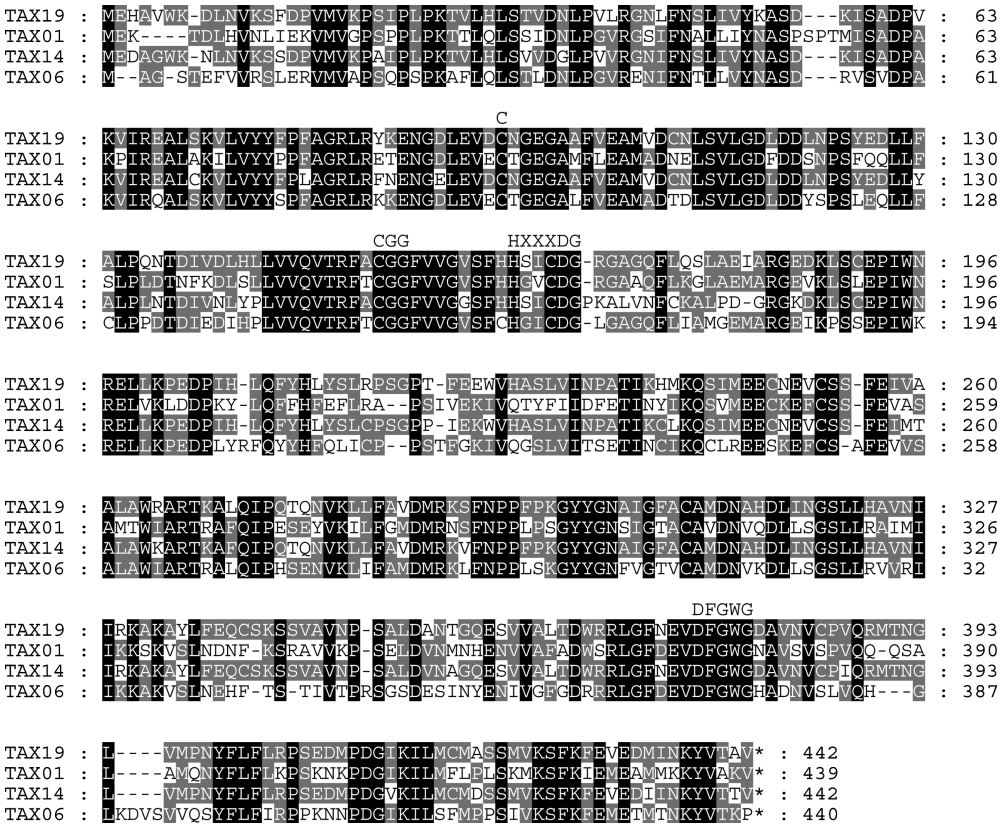


Fig. 3. Deduced amino acid sequence alignment of taxoid acetyltransferases: TAX19 (taxoid 5α-acetyltransferase), TAX1 (previously identified taxoid 5α-acetyltransferase), TAX14 (taxadien-5α,13α-diol and 5α,10β-diol acyltransferase), and TAX6 (10-DAB-10-*O*-acetyltransferase). Black boxes indicate identical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

[24] (TAX1 at 0.2‰ and TAX19 at 0.1‰) relative to the other acyltransferases of Taxol biosynthesis (all >1.0‰). These low abundances may result from downregulation of the TAX1 and TAX19 genes upon methyl jasmonate elicitation of the *Taxus* cells; alterations in gene expression upon induction of Taxol biosynthesis are not yet well defined.

Conclusions

With the acyl and aroyltransferases involved in Taxol biosynthesis seemingly defined [11,18,19], we turned our attention to the origin of other acylated taxoids, particularly the numerous acetylated derivatives, and functionally screened all of the available *Taxus* acyltransferase clones with the newly acquired taxoid test substrates using acetyl CoA as co-substrate. From this family of taxoid acyltransferases, a new clone, denoted TAX19, was found to possess taxadien-5α-*O*-acetyltransferase activity like that of the previously isolated TAX1 acetyltransferase [22]. Both TAX1 and TAX19 enzymes were subsequently shown to be capable of utilizing a range of polyhydroxylated taxoid substrates with differing regioselectivities, TAX1 with preference for C9 and C10 acetylation, and TAX19 with preference for C13 acetylation. The observation that these two enzymes are capable of acetylating taxadien-5α-ol with respectable kinetics suggests a role in vivo. Acetylation at C5 (i.e., following the first hydroxylation of the taxane core to taxadien-5α-ol [32]) would appear to be an early step of taxoid biosynthesis, the C5-acetylated product leading to subsequent hydroxylation at C10 [13] and establishing a structural element at C5 that is seemingly necessary for eventual construction of the oxetane D-ring [23] (see Fig. 2). Although taxadien-5α-yl acetate has been demonstrated as a metabolite of *Taxus* [33,38], and the enzymology of the acetylation of taxadien-5α-ol established [33] and the corresponding gene isolated [22], it has not been possible to adequately evaluate the intermediacy of taxadien-5α-yl acetate in taxoid biosynthesis by feeding studies with *Taxus* cells because the uptake of this metabolite is very inefficient.

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

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

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