



## Taxol biosynthesis: Identification and characterization of two acetyl CoA: taxoid-O-acetyl transferases that divert pathway flux away from Taxol production <sup>☆</sup>

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13-Acetyl-9-dihydro-baccatin III  
Taxusin

### ABSTRACT

Two cDNAs encoding taxoid-O-acetyl transferases (TAX 9 and TAX 14) were obtained from a previously isolated family of *Taxus* acyl/aroyl transferase cDNA clones. The recombinant enzymes catalyze the acetylation of taxadien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol to generate taxadien-5 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -tri-acetoxy-9 $\alpha$ -ol and taxadien-5 $\alpha$ ,9 $\alpha$ ,13 $\alpha$ -triacetoxy-10 $\beta$ -ol, respectively, both of which then serve as substrates for a final acetylation step to yield taxusin, a prominent side-route metabolite of *Taxus*. Neither enzyme acetylate the 5 $\alpha$ - or the 13 $\alpha$ -hydroxyls of taxoid polyols, indicating that prior acylations is required for efficient peracetylation to taxusin. Both enzymes were kinetically characterized, and the regioselectivity of acetylation was shown to vary with pH. Sequence comparison with other taxoid acyl transferases confirmed that primary structure of this enzyme type reveals little about function in taxoid metabolism. Unlike previously identified acetyl transferases involved in Taxol production, these two enzymes appear to act exclusively on partially acetylated taxoid polyols to divert the Taxol pathway to side-route metabolites.

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### Introduction

Yew (*Taxus*) species produce many diverse taxoids (taxane diterpenoids), including the anti-cancer drug Taxol (generic name paclitaxel), which has also recently gained application in the treatment of restenosis and Alzheimer's disease [1–5]. Due to the limited supply of the drug from its natural source, semi-synthetic procedures for the production of Taxol from more readily advanced taxoid precursors (e.g. 10-deacetylbaccatin III) have been developed [6,7]. For the foreseeable future, sourcing of the drug and its precursors will rely on isolation from yew species or derived cell cultures [8]. Therefore, a full understanding of the biosynthesis of Taxol and its related metabolites is mandatory for improving biological production techniques [9,10].

*Taxus* species produce about 400 different taxoids, all of which are based upon the unique taxane (pentamethyl [9.3.1.0]<sup>3,8</sup> tricyclopentadecane) skeleton [11]. Some 19 enzymatic steps are involved in Taxol biosynthesis, starting from the primary intermediate geranylgeranyl diphosphate [12] (Fig. 1), which, in the first

committed step of the pathway [13], is cyclized to taxa-4(5),11(12)-diene as the parent of all taxoids [14]. A series of cytochrome P450-mediated oxygenations, two acetylations, a benzylation, oxidation at C9 and oxetane formation leads to baccatin III, upon which construction of the *N*-benzoylphenylisoserine side chain at C13 completes the pathway to Taxol [15,16]. In addition to the acetates at the C4 and C10 positions of Taxol, acetyl functions can also be variously introduced at C1, C2, C7, C9, and C13 of the taxane core resulting in the great diversity of naturally occurring taxoids. These taxoid metabolites may represent defense compounds (antifeedants, antimicrobials, etc.) [17–19], but they also constitute significant side-routes that divert pathway flux away from Taxol to decrease production yields of the target drug.

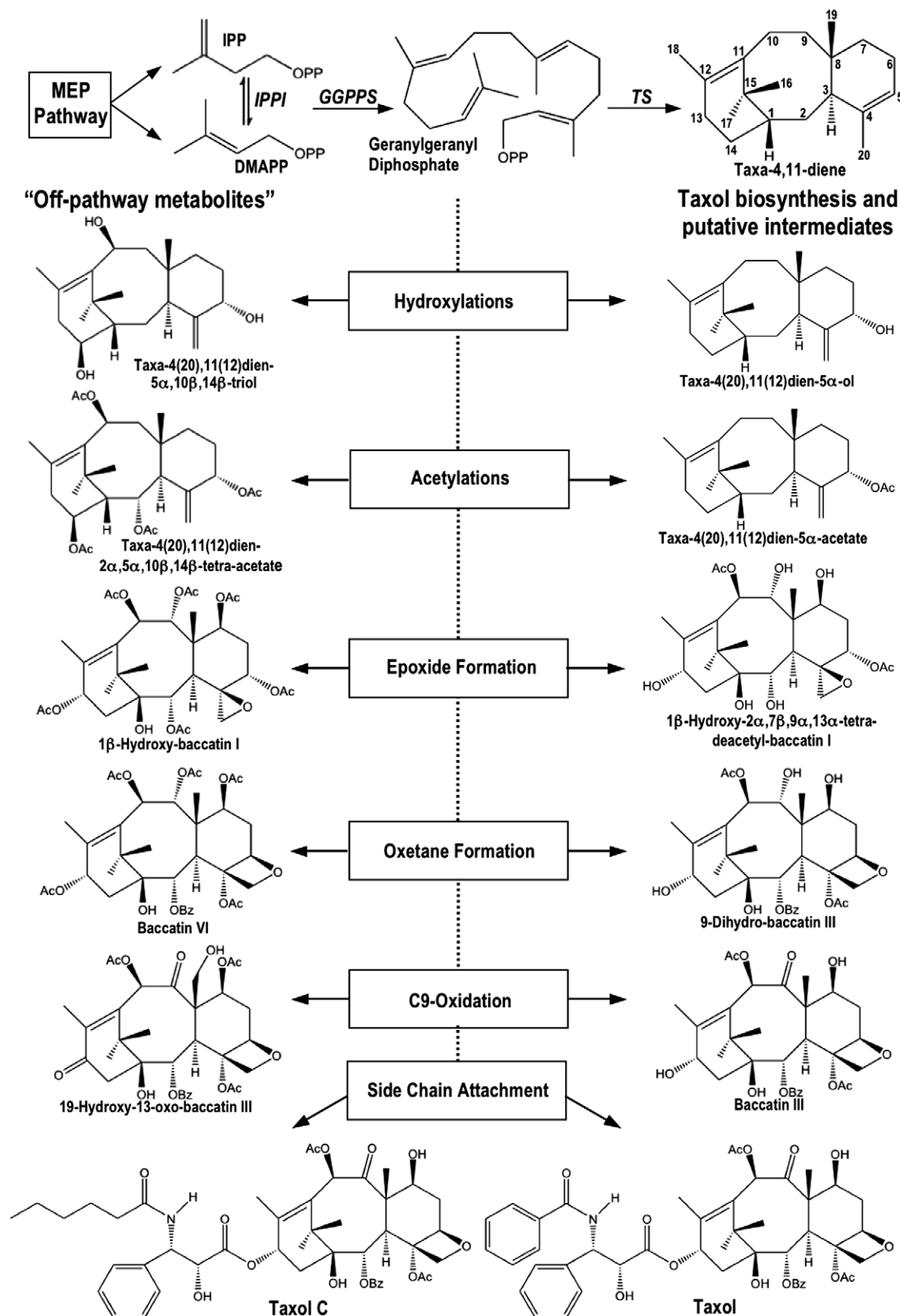
Many genes encoding relevant enzymes of the Taxol pathway have been identified and their functions characterized, including those for taxadiene synthase [20], several cytochrome P450 taxoid hydroxylases [21–25,26 for review] and all five of the presumptive acyl and aroyl transferases [27–32]. No acetyl transferases thought to be involved in “off pathway” steps have yet been confirmed, although it is known for the various transferases now characterized that regioselectivity of the acyl/aroyl transfer may be somewhat promiscuous, dependent on the structure of the test substrate, and can result in acylations of the taxane core at alternative positions (e.g. C9 and C13) [32,33]. The identification of “off pathway” acyl transferases could have important biotechnological application because knockout of these diversionary steps would be expected to increase the yields of Taxol and its relevant precursors.

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**Fig. 1.** Taxol biosynthetic pathway starting from primary metabolism and including proposed intermediates, as well as metabolites which are proposed not to lead to Taxol production. MEP, methylerythritol phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IPPI, isopentenyl diphosphate isomerase; GGPPS, geranylgeranyl diphosphate synthase; TS, taxadiene synthase. Figure modified from [48].

Several strategies have been applied for cloning genes encoding acyl and aroyl transferases from *Taxus* species [34,35], and have re-

sulted in the acquisition of a family of 16 closely related acyl/aroyl transferase clones, including the previously identified transferases

thought to be involved in Taxol biosynthesis [27–32]. In this paper, we report the identification and characterization of two new taxoid acetyl transferase clones (denoted TAX 9 and TAX 14), compare properties of the recombinant enzymes to previously identified taxoid-*O*-acetyl transferases, and discuss the role of these enzymes in taxoid metabolism.

## Materials and methods

### Substrates

The preparation of taxa-4(20),11(12)-dien-5 $\alpha$ -acetoxy-9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triol has been described previously [30 and references therein].

### Synthesis of taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol

Taxusin (taxa-4(20),11(12)-5 $\alpha$ ,9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -tetraacetate; 65.5 mg) was treated with sodium methoxide (2 mL; 0.5 N in diethyl ether) in THF (1 mL) at room temp. for 48 h, and the reaction was then quenched with 2 mL brine, and the product was extracted into diethyl ether (3  $\times$  3 mL). The combined organic fractions were partially purified by silica gel column chromatography with ethyl acetate to yield taxusin tetraol (taxa-4(20),11(12)-5 $\alpha$ ,9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -tetraol; 52.9 mg). The tetraol was stirred with 2,2-dimethoxypropane (2.8 mL) and *p*-toluene sulfonic acid (1.38 mg) in 5 mL DMF for 6 h at 67 °C to introduce the C9/C10-acetonide protecting group. The resulting acetonide (16.9 mg) was purified by preparative TLC (silica gel: hexane/acetone: 70/30 (v/v)) and then acetylated at C5 and C13 by treating with triethylamine (TEA; 203  $\mu$ L), 4-(dimethylamino)-pyridine (DMAP; 11.9 mg) and acetic anhydride (93  $\mu$ L) in CH<sub>2</sub>Cl<sub>2</sub> overnight at room temp. This reaction mixture was then extracted with ethyl acetate (3  $\times$  2 mL) and the material purified by silica gel column chromatography with ethyl acetate to obtain 15 mg of the diacetylated product. The acetonide group was then hydrolytically removed by overnight reflux in 1 mL 0.5 N HCl in THF, which was added dropwise during the first 6 h. The crude diacetate product was identified by comparison with an authentic standard and was purified by HPLC (fractions collected at 27.4–28.2 min; for HPLC conditions see below; NMR data for taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol see [24]).

### Heterologous expression in *E. coli* and functional screening

Random sequencing of a *Taxus* EST library [35] and homology-based screening of an induced *Taxus* cell cDNA library [34] yielded 16 new acyl/aroyl transferase clones which were originally subcloned into pSBET for functional screening [32,36]. This screen resulted in the identification of six clones of defined functions [15,16,27–32]. The remaining undefined clones were subcloned into pET32a<sup>+</sup> and individually transformed into *E. coli* BL21(DE3)-codon<sup>+</sup> (Stratagene, La Jolla, CA) competent cells for expression. This new construct introduces a 6-histidine-tag at the C-terminus to permit immobilized-metal affinity chromatographic (IMAC) purification [37]. Five of the remaining clones were transformed and grown overnight at 37 °C in 10 mL of Luria–Bertani (LB) medium containing ampicillin (100  $\mu$ g/mL), chloramphenicol (25  $\mu$ g/mL) and tetracycline (10  $\mu$ g/mL). These starter cultures were individually added to 1 L of the same supplemental media and grown at 37 °C until cell density reached  $A_{600}$  ~0.8. Expression was then induced by addition of 0.25 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG), and the cultures were maintained at 18 °C/275 rpm. After 18 h, the cells were harvested by centrifugation (25 min, 3000 g), resuspended in 15 mL lysis buffer (50 mM potassium phosphate, pH 7.0, containing 300 mM NaCl, 10 mM imidazole and 10 % (v/v) glycerol), and disrupted by sonication (4  $\times$  30 s at

medium power) on ice using a Virsonic 475 (Virtis, Gardiner, NY) with a 1/2 in. probe. The homogenates were centrifuged for 1 h at 42,000g to obtain the operationally soluble enzyme fractions. The resulting supernatants were filtered (Nalgene syringe filter SFCA, 0.45  $\mu$ m) to remove residual cell debris and partially purified by IMAC using a cobalt-charged resin consisting of tentacle polymethacrylate beads [37]. Functional screening was conducted with these partially purified enzyme preparations using available taxoid substrates as previously described [15,24,32]. Each substrate (100  $\mu$ M in CH<sub>3</sub>CN) was transferred to a 10 mL screw-capped vial, the solvent was evaporated, and 50  $\mu$ g of the protein was then added to the dried substrate, along with [<sup>3</sup>H]acetyl CoA (22  $\mu$ M, 0.5 mCi/mL, 200 mCi/mmol), 100  $\mu$ M unlabeled acetyl CoA and sufficient 25 mM MOPSO buffer (pH 7.25) to bring the final volume to 200  $\mu$ L. The reaction mixtures were incubated for 90 min at 31 °C, followed by addition of saturating NaCl, and extraction of the products and residual substrate with 3  $\times$  1 mL portions of diethyl ether. After evaporating the organic solvent, the resulting residue was redissolved in CH<sub>3</sub>CN and analyzed by radio-HPLC (Agilent 1100 Series HPLC coupled to a Packard A-100 flow-through radio-detector using 3a70b scintillation cocktail; RPI, Mt. Prospect, IL), employing a SUPELCO Discovery® HS F5 column (250  $\times$  4.6 mm, 5  $\mu$ m) with a solvent gradient H<sub>2</sub>O/CH<sub>3</sub>CN (95/5 to 0/100) over 50 min (flow rate: 1 mL/min).

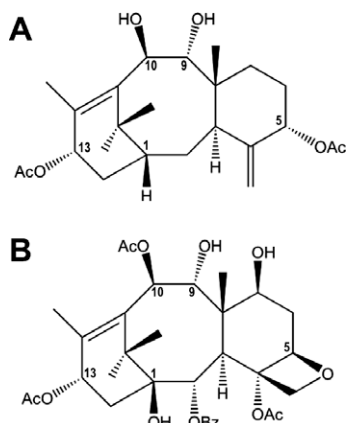
### Large-scale preparation of recombinant acetyl transferases

Large-scale (6  $\times$  1 L) cultures of *E. coli* BL21(DE3)codon<sup>+</sup> competent cells containing the target acetyl transferase genes (designated TAX 9 and TAX 14) were prepared and partially purified as described above. An additional purification step was introduced by eluting undesired protein (with 60 mM imidazole) from the IMAC column prior to elution of the recombinant transferase (with 250 mM imidazole). This procedure resulted in ~30% purity as determined by SDS/PAGE analysis [38] and western blotting [39]. The partially purified enzyme was dialyzed overnight (against 50 mM potassium phosphate, pH 7.0, containing 10% glycerol and 20 mM  $\beta$ -mercaptoethanol) at 4 °C, and then concentrated using an Amicon Ultra-4 NMWL 30 centrifugal filter unit (15 min, 3700 rpm). The resulting TAX 9 preparation was subsequently applied to a diethylaminoethyl-cellulose ion exchange column (5 g, Whatman DE-52, Clifton, NJ), previously equilibrated with 50 mM MOPSO buffer, pH 7.2, containing 5% (v/v) glycerol, 5 mM MgCl<sub>2</sub> and 0.5 M DTT. After removing unbound protein, the target enzyme was eluted with a linear NaCl gradient from 0 to 300 mM in equilibration buffer (100 mL total volume, 3 mL/min). Three milliliters samples were collected and analyzed by SDS/PAGE. Fractions containing the recombinant enzyme (0–45 mM) were combined, dialyzed and concentrated as described above, to provide the enzyme preparation (>95% purity) used for kinetic evaluation.

In the case of TAX 14, kinetic evaluations were carried out with partially purified protein obtained by the IMAC procedure (~25% purity) described above, because the additional chromatographic step provided no higher purity.

### Product identification and enzyme characterization

For kinetic evaluation, taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol (TAX 9 and TAX 14) and 13-acetyl-9-dihydrobaccatin III (TAX 9) were used as test substrates (Fig. 2). For structural identification of the products generated from taxadien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol, assays were scaled up to yield sufficient product for NMR analysis (~500  $\mu$ g). Following extraction as described above, the two products were purified by HPLC on an Alltech HYPERSIL BDS C18 5  $\mu$  column (250 mm  $\times$  4.6 mm) with



**Fig. 2.** Structures of TAX9 and TAX14 test substrates. (**A**, taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol; **B**, 13-acetyl-9-dihydro-baccatin III).

isocratic elution (H<sub>2</sub>O/CH<sub>3</sub>CN, 50/50 (v/v)). This procedure provided sufficient resolution of the two major products to permit subsequent identification as taxa-4(20),11(12)-dien-5 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triacetoxo-9 $\alpha$ -ol and taxa-4(20),11(12)-dien-5 $\alpha$ ,9 $\alpha$ ,13 $\alpha$ -triacetoxo-10 $\beta$ -ol. After evaporating the solvent from appropriate combined fractions, the resulting residue was redissolved in 100% CDCl<sub>3</sub> for NMR analysis. <sup>1</sup>H NMR, <sup>13</sup>C NMR, COSY, HMBC, HSQC and TOCSY were performed. <sup>1</sup>H NMR (taxadien-5 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triacetoxo-9 $\alpha$ -ol):  $\delta$ ; 0.7 (s, CH<sub>3</sub>), 1.20 (s, CH<sub>3</sub>), 1.7 (s, CH<sub>3</sub>), 1.69 (m, CH<sub>2</sub>), 1.75 (m, CH<sub>2</sub>), 1.80 (m, CH<sub>2</sub>), 1.83 (m, H1), 1.97 (s, CH<sub>3</sub>), 2.69 (m, CH<sub>2</sub>), 2.99 (d, H3,  $J$  = 6.0 Hz), 4.84 (d, H20,  $J$  = 1.5 Hz), 4.95 (d, H10,  $J$  = 10.3 Hz), 5.20 (d, H20,  $J$  = 1.2 Hz), 5.35 (s, H5), 5.76 (d, H9,  $J$  = 10.3 Hz), 5.90 (t, H13,  $J$  = 9.0 Hz). <sup>1</sup>H NMR (taxadien-5 $\alpha$ ,9 $\alpha$ ,13 $\alpha$ -triacetoxo-10 $\beta$ -ol):  $\delta$ ; 0.92 (s, CH<sub>3</sub>), 1.10 (s, CH<sub>3</sub>), 1.50 (s, CH<sub>3</sub>), 1.66 (m, CH<sub>2</sub>), 1.69 (m, CH<sub>2</sub>), 1.80 (H1), 1.87 (m, CH<sub>2</sub>), 2.1 (s, CH<sub>3</sub>), 2.67 (m, CH<sub>2</sub>), 2.98 (d, H3,  $J$  = 5.4 Hz), 4.25 (d, H9,  $J$  = 10.1 Hz), 4.82 (d, H20,  $J$  = 1.5 Hz), 5.19 (d, H20,  $J$  = 1.4 Hz), 5.35 (t, H5,  $J$  = 2.8 Hz), 5.86 (d, H10,  $J$  = 9.3 Hz), 5.89 (dd, H13,  $J$  = 10.2 Hz).

In the case of 13-acetyl-9-dihydro-baccatin III as substrate, low yields did not allow the isolation of sufficient material for structural identification of the three products formed. However, LC-MS analysis confirmed that all three products contained one additional acetyl group, and so were almost certainly the C1, C7 and C9 acetate derivatives (see Fig. 2).

Reaction linearity with respect to time and protein concentration was first established, and these conditions were used to determine the pH optimum of the recombinant enzymes. With taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol as substrate, TAX 9 revealed a pH optimum of 7.25 for the first acetylation (see below) using 200  $\mu$ M substrate, 100  $\mu$ M [<sup>3</sup>H]acetyl CoA (0.5 mCi/mL; 200 mCi/mmol) and 20 ng of the purified enzyme. These assays were incubated for 6 min at 31 °C in MES (pH 6.0–7.0), MOPSO (pH 7.0–8.0), TRICINE (pH 8.0–9.0) and CAPSO (pH 9.0–10) buffers over intervals of 0.5 and 0.25 pH units (pH 6.5–8.0), respectively. TAX 14 showed a pH optimum of 7.5 for the first acetylation (see below) using 3  $\mu$ g of the partially purified enzyme and the same substrate and co-factor concentrations as before. These assays were carried out for 3 min at 31 °C in MES (pH 6.0–7.0), MOPSO (pH 7.0–8.0), TRICINE (pH 8.0–9.0) and CAPSO (pH 9.0–10) buffers over intervals of 0.5 pH units.

Kinetic values for were determined by Eadie-Hofstee plotting (Enzyme Kinetics Pro™, ver. 2.36 SynexChem™, LLC). These experiments were conducted with a substrate concentration range of 10–500  $\mu$ M, using 20 ng protein (TAX 9) or 3  $\mu$ g (TAX 14), at the pH optimum in 25 mM MOPSO buffer, and at operational satura-

tion of [<sup>3</sup>H]acetyl CoA. The reported values are means  $\pm$  SD of triplicate determinations.

TAX 9 kinetic assays (300 ng protein) with 13-acetyl-9-dihydro-baccatin III (substrate concentrations range 0.1–1.0 mM) were carried out for 10 min at the pH optimum in 25 mM MOPSO buffer, and at operational saturation of [<sup>3</sup>H]acetyl CoA. The extraction protocol was modified such that the reaction mixtures were quenched with 3 mL diethyl ether, to which 0.5 mL basic brine (pH 8.5) was then added for 15 min at room temp. to hydrolyze residual [<sup>3</sup>H]acetyl CoA. The diethyl ether phase was back extracted with basic brine (3  $\times$  1 mL) to remove [<sup>3</sup>H]acetate (sodium salt), and the solvent was evaporated. The resulting residue was redissolved in minimum CH<sub>3</sub>CN and quantified by liquid scintillation counting.

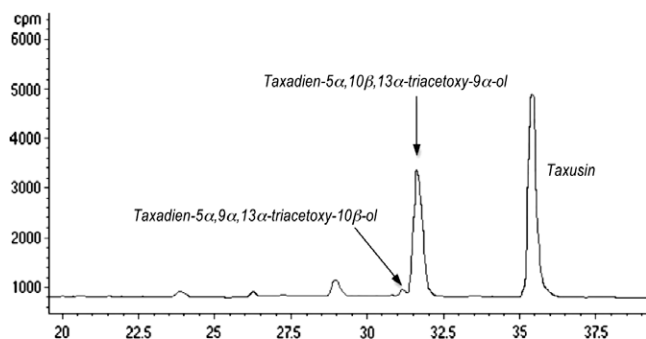
## Results and discussion

With the acyl and aroyl transferases of the Taxol biosynthesis pathway defined [16], recent attention has focused on “off pathway” transformations responsible for the formation of a large family of taxoids [11] bearing acetate groups at the C7, C9 and C13 positions of the taxane core, thus preventing progress towards Taxol and representing a significant diversion of pathway flux. Identification of the transferases involved in such pathway diversions has biotechnological implications, in that elimination of these alternative taxoid biosynthetic pathways should lead to an increase of the yields of Taxol.

Of the nine remaining undefined acyl/aroyl transferase clones from this previously acquired gene family [16], five were successfully subcloned into pET32a<sup>+</sup> and expressed in *E. coli*. The resulting recombinant proteins were partially purified by affinity chromatography and verified by SDS-PAGE and western blotting. The recombinant transferases were then screened under conditions previously established for taxoid-5 $\alpha$ -O-acetyl transferase (also used as a positive control) [31,32] using [<sup>3</sup>H]acetyl CoA and the following taxoids as test substrates: ( $\pm$ )-taxadien-5 $\alpha$ -ol; (+)-taxadien-5 $\alpha$ ,10 $\beta$ -diol; (+)-taxadien-2 $\alpha$ ,5 $\alpha$ -diol; (+)-taxadien-2 $\alpha$ ,5 $\alpha$ ,10 $\beta$ -triol; (+)-taxadien-5 $\alpha$ ,9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -tetraol and (+)-taxadien-5 $\alpha$ -acetoxy,9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triol. Of all the recombinant enzymes evaluated, two (designated TAX 9 and TAX 14) exhibited acetyl transferase activity, and only with (+)-taxa-4(20),11(12)-dien-5 $\alpha$ -acetoxy-9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triol as substrate. Two products were generated from this substrate in amounts too small to permit ready identification, but neither of which were the 5 $\alpha$ ,13 $\alpha$ -diacetoxy derivative nor the 5 $\alpha$ ,9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -tetraacetoxy derivative, as determined by lack of HPLC retention coincidence with the corresponding authentic standard. These results indicated that neither TAX 9 nor TAX 14 could acetylate the C13-position, that prior acetylation (at least at C5) was required for a functional substrate, and that the acetylation observed was almost certainly at the C9- and C10-positions.

To pursue the issues of substrate selectivity and regioselectivity of the TAX 9 and TAX 14 transferases, taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol was prepared as a test substrate, and the assay was repeated under the same conditions (neutral pH) followed by radio-HPLC analysis of the products as before. In this case, the TAX 9 recombinant enzyme gave rise to three products (Fig. 3) that were not present in the boiled enzyme control. Large-scale incubations and HPLC-based purification permitted the isolation of sufficient material for NMR analysis which confirmed the identities of the three products as taxa-4(20),11(12)-dien-5 $\alpha$ ,9 $\alpha$ ,13 $\alpha$ -triacetoxo-10 $\beta$ -ol, taxa-4(20),11(12)-dien-5 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triacetoxo-9 $\alpha$ -ol, and taxusin (the fully acetylated derivative). Similar analysis using the TAX 14 recombinant enzyme afforded the same three products, but, in this case, acetylation at the C9-position was preferred to yield principally taxa-4(20),11(12)-dien-





**Fig. 3.** HPLC-radio chromatogram of the products generated from TAX9 using taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol as substrate and [ $^3$ H]acetyl CoA as co-substrate. Assay conditions: 90 min at 31  $^{\circ}$ C and pH 7.0; taxusin appears as the main product due to the secondary acetylation of the primary products over time.

5 $\alpha$ ,9 $\alpha$ ,13 $\alpha$ -triacetoxy-10 $\beta$ -ol (product identities confirmed as before).

From these results, it would appear that the TAX 9 and TAX 14 transferases prefer as substrates taxoid polyols that are already partially acetylated, unlike two previously described taxoid acetyl transferases [31,32], and show regioselectivity for acetylation on the “northern hemisphere” of the taxane core at the C9- and C10-positions. Acetylation at C5 is considered to be an early step in the biosynthesis of Taxol, but acetylation at C13 (as in the test substrate taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol) can lead only to off pathway metabolites because construction of the normal C13 *N*-benzoyl phenylisoserine side chain of Taxol is blocked. Given the apparent preference of TAX 9 and TAX 14 for the 13 $\alpha$ -acetoxy taxoid substrate, it seems likely that these transferases serve to modify taxoid intermediates that are already destined for side-route accumulation. Thus, both TAX 9 and TAX 14 could play a role in the production of the tetraol tetraacetate taxusin, which is a major taxoid of yew heartwood [6 and references therein].

9,13-Diacetoxy-9-dihydro-baccatin III is a major taxoid often accumulated in *Taxus* cell cultures [49,50]. To determine if TAX 9 and TAX 14 could play a role in the production of this off pathway metabolite, both enzymes were evaluated as above, using 13-acetoxy-9-dihydro-baccatin III as substrate. Surprisingly, only TAX 9 (with apparent preference for acetylation at C10) exhibited catalytic activity in the production of three mono-acetylated products (as determined by LC–MS analysis). Product yields were too low to permit isolation and structural identification by NMR. Nevertheless, 13-acetyl-9-dihydro-baccatin III offers only three free hydroxyl functions at C1, C7 and C9; therefore, it would appear that acetylation (albeit inefficient) at all three positions was observed. Taxoids bearing acetate groups at C7 and C9 are common, but acetylation at C1 is rare [40]. In any event, it seems unlikely that TAX 9 plays a major role in the production of 9,13-diacetoxy-9-dihydro-baccatin III.

#### Enzyme characterization

Evaluation of transferase activity as a function of pH revealed that both TAX 9 and TAX 14 had a pH optimum between 7.25 and 7.50 (for the first acetylation), with half maximal velocities at approximately pH 6.0 and 8.3. Examination of product distribution as a function of pH demonstrated an alteration of product profile at alkaline pH. Thus, at pH 9.5 (with taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol as substrate), the fully acetylated product taxusin was the main product of TAX 9 rather than the triacetylated intermediate observed near neutral pH. With TAX 14 at pH 9.0 (with the same substrate), acetylation at the C9 $\alpha$ - and

C10 $\beta$ -hydroxyls occurs at comparable rates (the C9 $\alpha$ -hydroxyl is kinetically preferred at neutral pH), and the peracetylated product also accumulates. With taxa-4(20),11(12)-dien-5 $\alpha$ -acetoxy-9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triol as substrate at alkaline pH, product distribution was also somewhat altered for both enzymes. However, TAX 9 at pH 9.0 failed to produce taxusin, and with TAX 14, taxusin could only be detected as a minor product. Thus, for both enzymes, acetylation of the C13 $\alpha$ -hydroxyl was not favored under any pH condition.

Although the overall rate of acetyl transfer was somewhat suppressed at alkaline pH for both enzymes, it is clear that pH conditions do influence the regioselectivity of transfer to the vicinal C9 $\alpha$ - and C10 $\beta$ -hydroxyls of the taxane B ring. Such alternation in transfer selectivity may reflect changes in enzyme conformation or in the protonation state [43] of the active site histidine considered to play an essential role in acyl transfer catalysis [41,42].

TAX 9 and TAX 14 exhibited differences in  $K_M$  values with taxa-4(20),11(12)-dien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol as substrate at the pH optimum. With TAX 14, a  $K_M$  value of 19.5  $\mu$ M was observed, and with TAX 9 a  $K_M$  value of 400  $\mu$ M was noted. Rates of acetyl transfer were roughly comparable for the two enzymes (0.9–1.5  $s^{-1}$ ). With TAX 9 using 13-acetyl-9-dihydro-baccatin III as substrate, a  $K_M$  value of 180  $\mu$ M was determined, and the rate of acetyl transfer was reduced by a factor of about 10. Too few “off pathway” enzymes have been evaluated thus far to permit informed comparison with their Taxol pathway counterparts [16]; however, a preliminary assessment would suggest that there are no significant differences in kinetic behavior for the functional substrates tested and that many of both types of transferases are somewhat promiscuous with regard to substrate utilization and regioselectivity of the acylation.

#### Sequence analysis

The cDNAs for TAX 9 (1317 bp) and TAX 14 (1329 bp) encode a respective 438 residue (TAX 9) and a 442 residue (TAX 14) protein with calculated molecular weights (and pI values) of 48,414 (pI 6.1) (TAX 9) and of 48,970 (pI 5.6) (TAX 14). Neither cDNA appears to encode organellar-targeting information, and the size of the encoded enzymes is consistent with SDS–PAGE analysis and similar to that of previously identified taxoid acyl transferases [30–32].

The deduced amino acid sequences of TAX 9 and TAX 14 (Fig. 4) reveal the highly conserved HXXXDG catalytic triad (aa 164–169 (TAX 14) and 165–170 (TAX 9)) identified in other acetyl transferases [32,34,44–46]. Site-directed mutagenesis of the triad-histidine has proven the importance of this residue for catalysis by this enzyme type [45,46], and led to the hypothesis that this histidine acts as the general base in the transfer of the acyl group from acyl CoA to the substrate hydroxyl function. Wanatabe et al. [47] have also shown that a cysteine residue is important in catalysis by *O*-acetyl transferases, and alignment of the taxoid acetyl transferases reveals two conserved cysteines in addition to that of the HXXCDG motif (aa 96 (TAX 14) and 97 (TAX 9), and aa 153 (TAX 14) and 154 (TAX 9) as part of the CGG motif [45]. The protein sequences of the taxoid acetyl transferases also reveal the DFGWG motif (aa 374–378 of TAX 9 and 375–379 of TAX 14), which is another highly conserved element of the acetyl transferases [45].

TAX 9 and TAX 14 show moderate sequence similarity and identity scores of 73% (S) and 62% (I). The multiple protein sequence alignment of TAX 9, TAX 14 and the previously described *Taxus* acetyl transferases also shows comparable identity scores (~60%). As previously reported [32], TAX 14 and TAX 19 reveal significantly higher homology (87% I, 93% S), even though TAX 14 is now considered to be an “off pathway” contributor, whereas TAX 19 appears to be responsible for the first acetylation (at C5 $\alpha$ ) of

|       |   |                                                                 |   |     |
|-------|---|-----------------------------------------------------------------|---|-----|
| TAX1  | : | ---MEKTDLHVNLIEKVMVGSPPLPKTTLQLSSIDNLPVGRGSIFNALLIYNASPSPT      | : | 56  |
| TAX6  | : | ---MAGSTEFVVRSLERVMVAPSQSPKAFQLQLSTLDNLPGVRENIFNTLLVYNASDR--    | : | 55  |
| TAX9  | : | MEKAGSSTEFHVKISDPVMVPPCIPSPKTIQLSAVDNYPVVRGNILDCLLVYNASNT--     | : | 58  |
| TAX14 | : | -MEDAGWKNLNVKSSDPVMVKPAIPLPKTVLHLSVVDGLPVVRGNIFNSLIYVNASDK--    | : | 57  |
| TAX19 | : | -MEHAVWKDLNVKSFDPVMVKPSIPLPKTVLHLSVVDNLPVLRGNLFNSLIYVKASDK--    | : | 57  |
| TAX1  | : | MISADPAKPIREALAKILVYYPFAGRLRETENGDLVEVCTGEGAMFLEAMADNELSVLG     | : | 116 |
| TAX6  | : | -VSVDPAKVIRQALSKVLVYYPFAGRLRKKENGDLVEVCTGEGALFVEAMADTDLVLG      | : | 114 |
| TAX9  | : | -ISADPATVIREALSKVLVYYPFAGRMRNKGDELEVDCTGEGALFVEAMADDNLSVLG      | : | 117 |
| TAX14 | : | -ISADPAKVIREALCKVLVYYPFAGRLRFNENGELEVDGNGEGAAFVEAMVDCNLSVLG     | : | 116 |
| TAX19 | : | -ISADPVKVIREALSKVLVYYPFAGRLRYKENGDLVEVDGNGEGAAFVEAMVDCNLSVLG    | : | 116 |
| TAX1  | : | DFDDSNPSFQQLLFSLPLDTNFKDLSLLVVQVTRFTCGGFVVGVSFHHGVCDG- RGAQF    | : | 175 |
| TAX6  | : | DLDDYSPSLEQLLFCLPPDIEDIHLPLVVQVTRFTCGGFVVGVSFCHGICDG- LGAGQF    | : | 173 |
| TAX9  | : | GFDYHNPAFGKLLYSPLDTPHDLHPLVVQVTRFTCGGFVVGSLDHSICDG- RGAGQF      | : | 176 |
| TAX14 | : | DLDDLNPSEYEDLLYALPLNTDIVNLYPLVVQVTRFACGGFVVGGSFHHHSICDGPKALVN   | : | 176 |
| TAX19 | : | DLDDLNPSEYEDLLFALPQNTDIVDLHLLVVQVTRFACGGFVVGVSFHHHSICDG- RGAGQF | : | 175 |
| TAX1  | : | LKGLAEMARGEVKLSLEPIWNRELKLDLPKY-LQFFHFEFLRAP-SIVEKIVQTYFIID     | : | 233 |
| TAX6  | : | LIAMGEMARGEIKPSSEPIWKRELLKPEDPLYRFQYHFLICPP-STFGKIVQGSVLIT      | : | 232 |
| TAX9  | : | LKALAEMARGEAKPSLEPIWNRELKPEDLIR-LQFYHFESMRPP-PIVEEVQASIVN       | : | 234 |
| TAX14 | : | CKALPD-GRGDKLSCEPIWNRELKPEDPIH-LQFYHLYSLCPSPGPIEKVWVHASLVIN     | : | 234 |
| TAX19 | : | LQSLAEIARGEDKLSCEPIWNRELKPEDPIH-LQFYHLYSLPPSGPTFEVWVHASLVIN     | : | 234 |
| TAX1  | : | FETINYIKQSVMEECKEFCSSEFEVASAMTWIARTRAFQIPSEYVKILFGMDMRNSFNPP    | : | 293 |
| TAX6  | : | SETINCIKQCLREESKEFCSAFEVVSALAWIARTRALQIPHSENVKLI FAMD MRKLFNPP  | : | 292 |
| TAX9  | : | SETISNIKQYIMEECKESSFAFEVVAALAWLARTRAFQIPHTENVKLLFAVDTRRSFDPP    | : | 294 |
| TAX14 | : | PATIKCLKQSIMEECNEVCSSFEIMTALAWKARTKAFQIPQTQNVKLLFAVDMRKVFNPP    | : | 294 |
| TAX19 | : | PATIKHMKQSIMEECNEVCSSFEIVAALTWRARTKALQIPQTQNVKLLFAVDMKKSFNPP    | : | 294 |
| TAX1  | : | LPSGGYGNISIGTACAVDNVQDLLSGSLLRAIMI IKKSKVSLNDNFK-SRAVVKPSELVDN  | : | 352 |
| TAX6  | : | LSKGGYGNFVGTVCAMDNVQDLLSGSLLRVRI IKKAKVSLNEHFT-STIVTPRSGSDES    | : | 351 |
| TAX9  | : | LPKGYYGNAAGNACAMDNVQDLLNGSLLRAVMI IKKSKVSLNENIR-AKTVMRPSAIDVN   | : | 353 |
| TAX14 | : | FPKGYYGNAIGFACAMDNAHDLINGSL LHAVNIIRKAKAYLFEQCSKSSVAVNPSALDN    | : | 354 |
| TAX19 | : | FPKGYYGNAIGFACAMDNAHDLINGSL LHAVNIIRKAKAYLFEQCSKSSVAVNPSALDN    | : | 354 |
| TAX1  | : | MNHENVVAFADWSRLGFDEVD FGWGNAVSVSPVQ--QSALAMQNYFLFLKPSKNKPDGI    | : | 410 |
| TAX6  | : | INYENIVGFGDRRLGFDEVD FGWGHADNVSLVQHGLKDVSVVQSYFLFIRPPKNNPDGI    | : | 411 |
| TAX9  | : | MKHESTVGLSDRLHGLGFNEVD FGWGDALNASLVQHG----VIQQNYFLFLQPSKNMNGGI  | : | 409 |
| TAX14 | : | AGQESVVALTDWRRGLGFNEVD FGWGDAVNVCPVQR-MTNGLVMPNYFLFLRPSEDMPDGV  | : | 413 |
| TAX19 | : | TGQESVVALTDWRRGLGFNEVD FGWGDAVNVCPVQR-MTNGLVMPNYFLFLRPSEDMPDGI  | : | 413 |
| TAX1  | : | KILMFLPLSKMKSFKIEMEAMMKYVAKV                                    | : | 439 |
| TAX6  | : | KILSFMPPSIVKSKFKFEMETMTNKYVTKP                                  | : | 440 |
| TAX9  | : | KIAMFMPQSKVKPFKIEMEALISKYATKV                                   | : | 438 |
| TAX14 | : | KILCMDSSMVKSFKFEVEDIINKYVTTV                                    | : | 442 |
| TAX19 | : | KILCMASSMVKSFKFEVEDMINKYVPAV                                    | : | 442 |

**Fig. 4.** Deduced amino acid sequence alignment of taxoid acetyl transferases: taxoid 5 $\alpha$ -acetyl transferase (TAX 1), 10-DAB-10-O-acetyl transferase (TAX 6), taxoid 10 $\beta$ -acetyl transferase (TAX 9), taxoid 9 $\alpha$ -acetyl transferase (TAX 14), and taxoid 5 $\alpha$ -acetyl transferase (TAX 19). Conserved motifs are highlighted.

the Taxol pathway. Moreover, while TAX 9 and TAX 14 do not share outstanding homology, both enzymes are active towards two common substrates (taxadien-5 $\alpha$ -acetoxy-9 $\alpha$ ,10 $\beta$ ,13 $\alpha$ -triol, taxadien-5 $\alpha$ ,13 $\alpha$ -diacetoxy-9 $\alpha$ ,10 $\beta$ -diol). These findings support the conclusion that the primary structure of this enzyme type does not permit inference about substrate utilization or the role of the enzyme in taxoid metabolism.

## Conclusions

While the identification of acyl and aroyl transferases involved in Taxol biosynthesis has been a major focus for several years [16], exploring “side-route” and “dead-end” reactions has gained in significance as these metabolic steps might be modified as an alternative approach to improving Taxol production. Taxol is but one of very many taxoids produced by *Taxus* species, and it is often a minor accumulated product. The designation of “off pathway” is thus of only operational value in describing those taxoid metabolic pathways not directed to the target drug. The concept of “off path-

way”, while artificial, is of biotechnological significance, because defining and modulating these steps has implications for redirecting pathway flux towards Taxol to increase drug yields, especially in *Taxus* cell cultures that are amenable to transgenic manipulation [51].

Functional screening of the remaining *Taxus* acyl transferase clones with the available taxoid test substrates revealed two new transferases, denoted TAX 9 and TAX 14, which, while inactive with Taxol pathway intermediates, were capable of acetylating the C9 $\alpha$ - and C10 $\beta$ -hydroxyls of the corresponding 5 $\alpha$ ,13 $\alpha$ -diacetylated tetraol. TAX 9 and TAX 14 were incapable of acetylating the C5 $\alpha$ - or C13 $\alpha$ -hydroxyls of taxol polyols, in contrast with the previously identified TAX 19, which showed preferences of these east-west pole positions and not the northern hemisphere 9 $\alpha$ /10 $\beta$ -vicinal hydroxyls [32]. Like other acetyl transferases presumed to function in “off pathway” transformations, TAX 9 and TAX 14 are somewhat promiscuous in substrate utilization and clearly promiscuous in 9 $\alpha$ /10 $\beta$  regioselectivity which is additionally pH-dependent. That TAX 9 and TAX 14 prefer, as substrates, polyols acetylated at C13, and thus incapable of progression to Taxol, and can acetylate the

9 $\alpha$ -hydroxyl, thus preventing oxidation to the ketone function of Taxol, implicate these two transferases in “off pathway” taxoid metabolism, and suggest that they play a role in the biosynthesis of the peracetylated tetraol taxusin, a prominent metabolite of *Taxus* species. Attempts to suppress TAX 9 and TAX 14 gene expression in *Taxus* cell cultures are in progress.

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