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# ACETYL CoA:10-DEACETYLBACCATIN-III-10-*O*-ACETYLTRANSFERASE ACTIVITY IN LEAVES AND CELL SUSPENSION CULTURES OF *TAXUS CUSPIDATA*

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**Key Word Index**—*Taxus cuspidata*; taxaceae; leaves; cell culture; acetyl-CoA:10-deacetylbaccatin-III-10-O-acetyltransferase; baccatin-III.

**Abstract**—Partially purified acetyl CoA:10-deacetylbaccatin-III-10-orthoxy-acetyltransferase from leaves and 3-year-old cell suspension cultures of *Taxus cuspidata* yields baccatin-III from 10-deacetyl-baccatin-III in the presence of acetyl-CoA. The enzyme is substrate selective and does not significantly catalyze the conversion of 10-deacetyltaxol to paclitaxel (Taxol®). Ammonium sulfate precipitations and anion exchange column chromatography yielded partially purified enzyme from *Taxus cuspidata* leaves and cell suspensions. The 0–40% ammonium sulfate-precipitated protein fraction showed consistent and significant enzyme activity. Cell culture-protein extracts yielded higher activities of the enzyme than did leaf-protein extracts. 1 ml aliquots of enzyme preparation with 150 nmol of 10-deacetylbaccatin-III and 4.4 nmol of [1-$^{14}$C]acetyl CoA for 1 h at 30°C produce approximately 1.1 pmol baccatin-III per hour per mg of protein. The products of the reactions were eluted using photodiode array HPLC and fractions containing baccatin-III or paclitaxel were collected and scintillation counted to determine the amount of radiolabeled product formed. Identity of product peaks was confirmed by retention time, photodiode array UV spectrophotometry and co-chromatography with authentic standards. 

## INTRODUCTION

The development of paclitaxel (Taxol®) as an approved chemotherapeutic agent in the treatment of ovarian and breast cancers has prompted intense efforts to improve our understanding of the total synthesis and biosynthesis of the compound. Studies on enzymes leading to paclitaxel and its precursors are a matter of considerable importance in improving our understanding of the fundamental mechanisms governing the biosynthesis of these complex molecules. Considerable progress has been achieved in the elucidation of paclitaxel biosynthesis. Initial studies found phenylalanine to be a precursor for the side-chain of taxoids in *Taxus baccata* [1]. More recently, it has been established that the assembly of the side-chain of paclitaxel from phenylalanine occurs via β-phenylalanine [2]; the side-chain attachment occurs as phenylisoserine followed by benzoylation of *N*-debenzoyltaxol [3]. Furthermore, it has been demonstrated that the first committed intermediate in taxoid biosynthesis is taxa-4(5),11(12)-diene and the enzyme catalyzing this slow reaction, taxadiene synthase, was purified and characterized (Ref. [4] and references therein). A cDNA encoding taxadiene synthase was cloned and sequenced [5], opening the possibility of generating transformed plants and cell cultures with altered paclitaxel production. However, studies on taxadiene activity in relation to paclitaxel production in *Taxus canadensis* cell suspension cultures have indicated that the rate-limiting steps on taxol biosynthesis lay farther down the pathway than the cyclization step [6].

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The first oxygenation step of taxa-4(5),11(12)-diene to taxadienol has been shown to be catalyzed by a cytochrome P450 (heme thiolate protein) from the microsomal fraction of stem and cultured cells of *Taxus*. The hydroxylation step appears to be slow relative to subsequent reactions [7]. Studies in *Taxus canadensis* have indicated an origin of the taxane ring from acetate and mevalonate, although rates of incorporation were relatively low [8]. Detailed studies on the biosynthesis of taxuyunnanine C (2$\alpha$, 5$\alpha$, 10$\beta$, 14$\beta$-tetra-acetoxy-4(20), 11-taxadiene) in cell suspensions of *Taxus chinensis* have shown that, at least in this system, the taxane skeleton does not originate from mevalonate as might be expected and may result from the condensation of a triose phosphate with activated acetaldehyde followed by a subsequent skeletal rearrangement of the product as proposed for isoprenoid biosynthesis in certain bacteria [9]. It has recently been demonstrated that root- and cambium-protein extracts of *T. baccata* catalyze the acetylation of 10-deacetylbaccatin-III with acetyl CoA [10]. We now describe the acetylation of 10-deacetylbaccatin-III (10-DAB) to baccatin-III by a partially purified enzyme derived from leaves and 3-year-old cell suspension cultures of *Taxus cuspidata*.

## MATERIAL AND METHODS

### *Plant material*

Leaves of *Taxus cuspidata* were harvested from four female trees approximately 2 m in height growing outdoors at the St. George Campus of the University of Toronto, Toronto, Canada. Taxonomic identification and herbarium data are as in Fett-Neto and DiCosmo [11]. Immediately post-harvest, the leaves were kept at 2°C, weighed and quick frozen in liquid nitrogen and maintained at −80°C until protein extraction occurred. Cell suspension cultures of *Taxus cuspidata* were grown in B5C2 medium, as previously described [12]. Suspensions were approximately 3 years old and had been routinely subcultured every 3 weeks. Cell suspensions in stationary phase were filtered through Whatman number 1 filter paper, weighed, quick frozen in liquid nitrogen and maintained at −80°C until protein extraction.

### *Protein extraction*

Protein extracts were prepared from approximately 500 g of fresh weight of leaves and 400 g of fresh weight of cell cultures. All protein extraction and purification procedures were carried out at 4°C in a walk-in cold chamber. Frozen leaves and cells were made into a fine powder using a blender, mortar and pestle and fine quartz (Sigma) as an abrasive agent. Protein was extracted in potassium phosphate buffer (0.05 M, pH 7.0) with 5% (w/v) of insoluble polyvinylpolypyrrolidone. The following were included in the extraction buffer: 0.05 mM EGTA, 0.5 mM phenylmethyl sulphydryl fluoride, 1 $\mu$g/ml leupeptin, 1.5 $\mu$g ml$^{-1}$ chymostatin and 0.5 mM of dithiothreitol and 10 mM ascorbate. The extraction buffer with plant material, approximately 3 ml of buffer per g of leaves/cells, was mixed for approximately 30 min, filtered through 10 layers of cheesecloth and centrifuged (15,000*g* for 20 min) to remove particulate debris.

### *Enzyme preparation*

The cell-free crude extracts were fractionated by ammonium sulphate (AS) precipitation initially with two concentrations: 0–40% (w/v) and 40–70% (w/v), respectively. Subsequently, most of the acetylation activity was associated with the 25–40% AS-precipitated fraction. Precipitation of each fraction was done for 2 h with gentle stirring. Resultant fractions were centrifuged (15,000*g* for 20 min) and the pellets were resuspended in ice-cold extraction buffer. The extracts were then applied to a DEAE-BIOGEL A column (2.5 × 35 cm). The protein was eluted using a stepwise NaCl gradient that used 40 ml steps that increased from 0.02 to 0.40 M NaCl in 0.02 M increments. The column had a flow rate of 1.0 ml/min and the eluent was collected in 10 ml fractions that were appropriately desalted, ultrafiltered and tested for enzyme activity.

### *Enzyme activity assay*

Enzyme activity was determined after 1 h at 30°C by incubating 4.4 nmol of [1-$^{14}$C]-acetyl CoA (55.0 mCi mmol$^{-1}$) and 50 or 150 nmol of 10-deacetylbacatin III (10-DAB) with protein extract in a final volume of 1 ml with constant mixing. Protein concentrations were determined according to Bradford [13]. Controls included: 1 ml of extraction buffer alone, or extraction buffer containing the following: heat denatured protein, no acetyl CoA and no 10-DAB. Reactions were terminated by the addition of 1 ml of dichloromethane for taxane extraction. Enzyme activity is expressed as pmol of product formed per mg protein per h.

### *Taxane extraction and analysis*

Taxanes produced in the assay mixture were extracted in dichloromethane (1:1 v/v) by vortex mixing for 1 min, followed by sonication for 10 s. Samples were then centrifuged at 2000*g* for 30 min at 20°C for improved phase separation. The top aqueous layer was removed and discarded. The organic layer was vacuum dried, resuspended in exact volume of HPLC grade methanol (75 or 150 $\mu$l). Particulates were removed using either a 0.45 $\mu$m filter or by centrifugation (14,000 rpm for 5 min) prior to HPLC analysis.

HPLC analysis of samples was done using a Hewlett-Packard HP1090A HPLC instrument

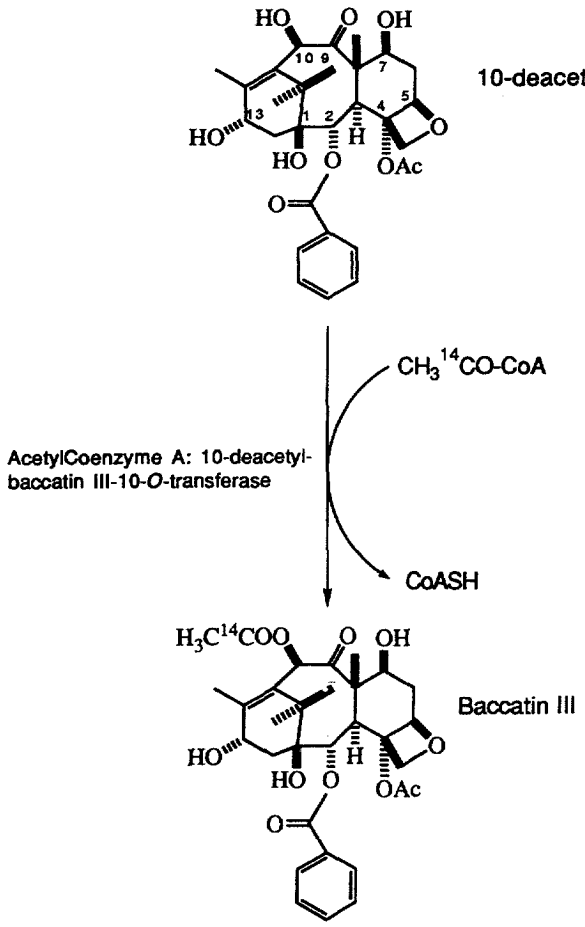


Fig. 1. The acetylation of carbon 10 in 10-deacetylbaccatin-III with [1-$^{14}$C] acetyl CoA by acetyl CoA: 10-deacetylbaccatin-III-*O*-acetyltransferase. Details of the reaction mixture are presented in the text.

equipped with photodiode array detection. Product separation was on a reverse-phase analytical column (Pierce RP-18 Spheri-5, 220 × 4.0 mm, 5 μm), with a solvent flow-rate of 1.0 ml min$^{-1}$. The mobile phase and the linear gradient elution profile started with MeOH–$H_2O$–MeCN (solvent A: 20:67:13 v/v) and ended with MeOH–$H_2O$–MeCN (solvent B: 20:27:53 v/v) within 50 min and included a 5 min wash in both solvents to restore the initial condition [14]. Injection volumes were 15 μl. Taxane quantification was done by the method of external standards with authentic paclitaxel and baccatin-III. Identity of baccatin-III was confirmed by retention time, UV spectra, co-chromatography with authentic standard and peak purity check by photodiode array spectrophotometry. Peaks corresponding to product eluted from the column were collected and examined for radioactive label by counting in a Beckman Liquid Scintillation Counter. Specific activities were calculated after correcting for background and counting efficiency.

*Statistical analysis*

Complete determinations of enzyme activity (incubation, product extraction, quantification, identification, label determination and activity calculation) for controls and samples were done in triplicate. Standard deviations are shown in the graphs. At least two completely independent experiments (two independent protein extractions from both needles and cell suspensions) were done with similar results.

## RESULTS

The activity of acetyl CoA: 10-deacetylbaccatin-III-10-*O*-transferase (Fig. 1) was investigated with protein extracts from leaves and cell suspensions of *Taxus cuspidata*. Initial enzyme activity experiments with non-radioactive acetyl CoA suggested the presence of 10-DAB acetyltransferase in leaf-protein extracts and in protein extracts from cell suspen-

sions (data not shown). For both protein extracts, activity appeared consistently in the 0–40% ammonium sulphate precipitated protein fraction, with insignificant or undetectable activity associated with the 40–70% ammonium sulphate precipitated protein fraction. The experiments with [1-$^{14}$C]-acetyl CoA and fresh needle protein extract confirmed 10-DAB acetyltransferase activity in the 0–40% protein fraction, yielding approximately 1.1 pmol of baccatin III per hour per mg of protein. Considerably lower 10-DAB acetyltransferase activity (approximately 0.15 pmol of baccatin III per hour per mg of protein) was observed in the 40–70% protein fraction. Activity in the 0–40% protein fraction was significantly higher than the activity of the various controls: no enzyme (CB), denatured enzyme (CD), no acetylCoA (CA) and no added 10-DAB (CS). The control without 10-DAB (CS) displayed higher activity relative to other controls. Repeated attempts to detect significant 10-deacetyltaxol acetyltransferase activity in the 0–40% protein fraction were not successful. An additional experiment confirmed the activity of 10-DAB acetyltransferase in both needle and cell suspension protein extracts of *Taxus cuspidata*. Activities were determined with the 25–40% ammonium sulphate-precipitated protein fraction from both sources, 50 nmol of 10-DAB and 4.4 nmol of [1-$^{14}$C] acetyl CoA. Leaf-protein extracts yielded approximately 0.18 pmol of baccatin III per hour per mg of protein [Fig. 2(a)], whereas cell suspension protein extracts displayed increased activity of approximately 0.27 pmol of baccatin III per hour per mg of protein [Fig. 2(b)]. Again, in this experiment a trend toward higher activity of the control without added substrate (CS) relative to other controls was observed.

Considering the 0–40% ammonium sulphate precipitated protein as the crude extract, the partial purification scheme applied resulted in a purification of 100-fold of 10-DAB acetyltransferase, with the total protein being reduced by a factor of 39. The specific activity doubled after the 25–40% ammonium sulphate precipitation step with the bulk of the specific activity increase occurring after the DEAE column fractionation.

## DISCUSSION

The occurrence of 10-DAB acetyltransferase activity, responsible for baccatin-III biosynthesis, in both leaves and cell suspension protein extracts from *Taxus cuspidata* is in good agreement with the presence of both baccatin-III and paclitaxel in these plant materials. The relatively higher 10-DAB acetyltransferase activity in cell suspension extracts

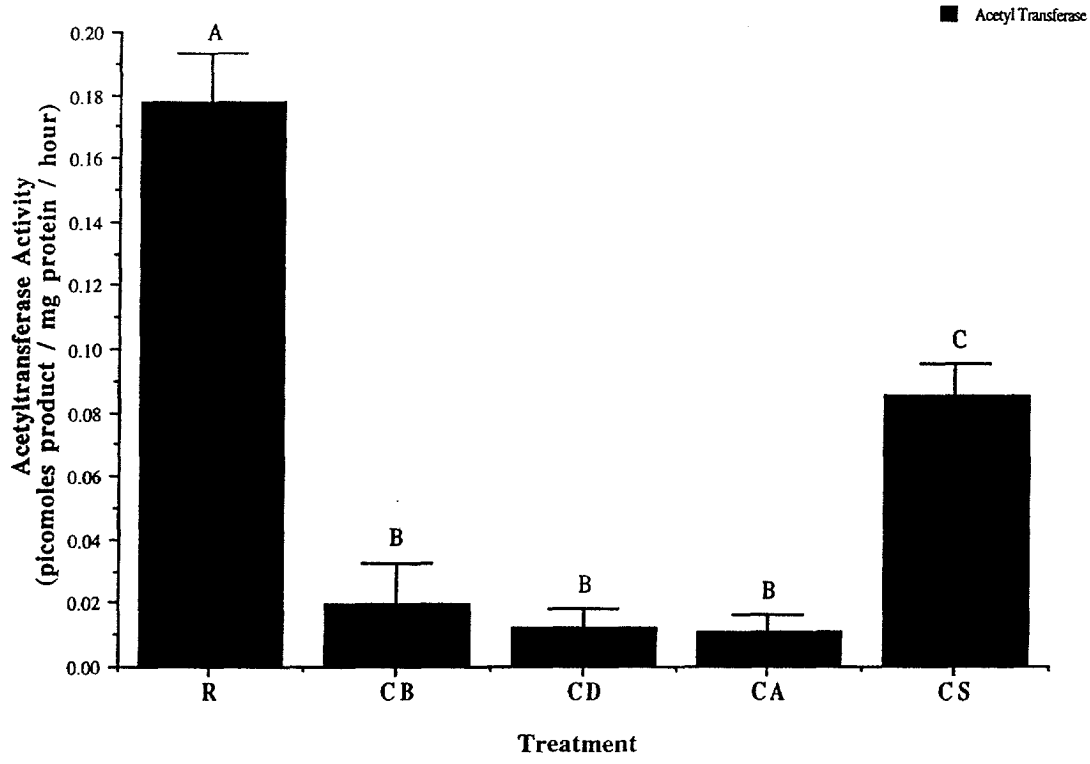


Fig. 2a—*Caption opposite*

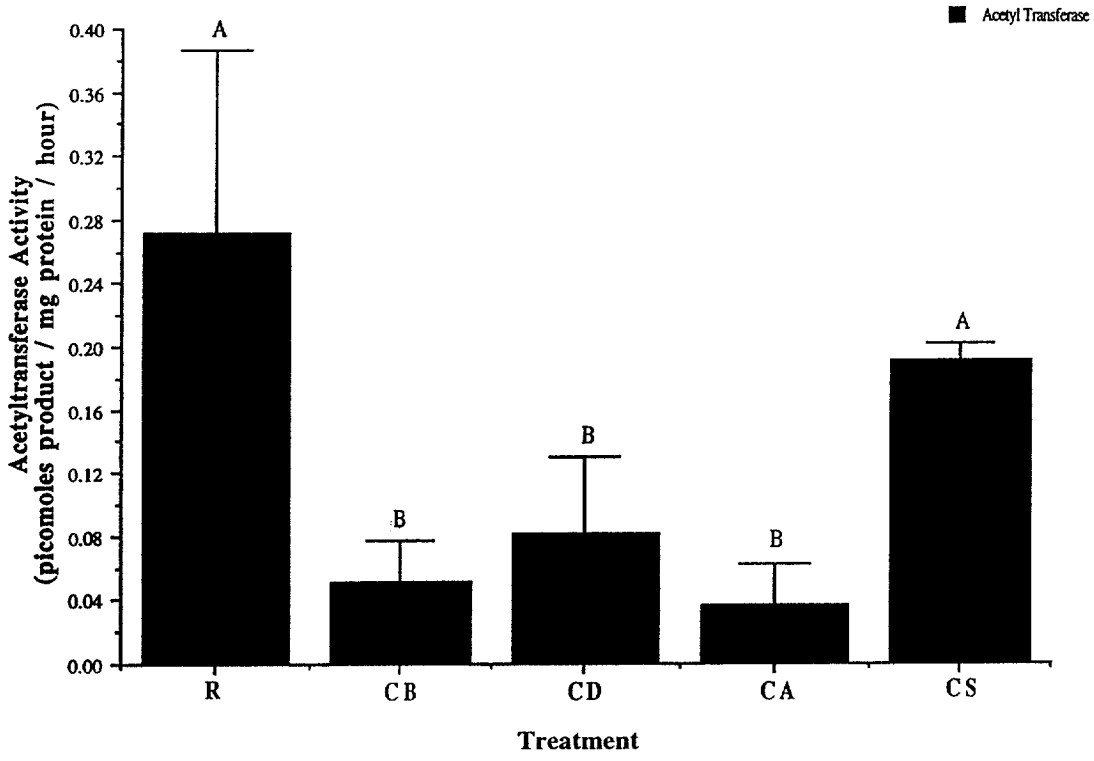


Fig. 2. (a) 10-deacetylbaccatin-III-*O*-acetyltransferase activity using the 25–40% ammonium sulfate-precipitated protein extracts from *Taxus cuspidata* leaves. The reaction mixture contained 1 ml of protein, 50 nmol of 10-DAB and 4.4 nmol [1-$^{14}$C] acetyl CoA. Control assays included: 1 ml buffer: without protein (CB) or with denatured protein (CD) or with no [1-$^{14}$C] acetyl CoA (CA) or with no 10-DAB (CS). All assays were incubated for 1 h at 30°C. Each condition was assayed in triplicate. Error bars are one standard deviation. Bars showing the identical letter designation do not differ at $p = 0.05$ by a Student's *t*-test. (b) 10-deacetylbaccatin-III-*O*-acetyltransferase activity using the 25–40% ammonium sulfate-precipitated protein extracts from *Taxus cuspidata* cell suspensions. The reaction mixture contained 1 ml of protein, 50 nmol of 10-DAB and 4.4 nmol [1-$^{14}$C] acetyl CoA. Control assays included: 1 ml buffer: without protein (CB) or with denatured protein (CD) or with no [1-$^{14}$C] acetyl CoA (CA) or with no 10-DAB (CS). All assays were incubated for 1 h at 30°C. Each condition was assayed in triplicate. Error bars are one standard deviation. Bars showing the identical letter designation do not differ at $p = 0.05$ by a Student's *t*-test.

compared to leaf extracts may partly explain the larger baccatin III/paclitaxel ratio in the cell culture line examined, which may also be related to putative lesion(s) downstream in the pathway leading to paclitaxel [12]. In addition, the fact that leaves tend to yield considerably higher amounts of paclitaxel compared to the cell cultures used in the experiments (0.025–0.035% vs 0.0004–0.0014% of the extracted dry weight, respectively) suggests that 10-DAB acetyltransferase is not a rate-limiting enzyme in paclitaxel biosynthesis; alternatively, this fact could be related to specific characteristics of paclitaxel biosynthesis in the cell culture line examined. Previously, 10-DAB acetyltransferase activity was detected in crude extracts from roots and cambium of stems of *T. baccata* [10]; higher activity of acetyltransferase was detected in root-protein extracts, the organ that also displayed the higher levels of paclitaxel. The significance of the correlation to the contribution of 10-DAB acetytransferase to the flux of taxane biosynthesis requires more detailed investigation.

Our results suggest that 10-DAB acetyltransferase is specific for 10-deacetylbaccatin-III; the enzyme failed to catalyze the acetylation of 10-deacetyltaxol to paclitaxel in any significant amount. The lower 10-DAB acetyltransferase activity observed when using 50 nmol 10-DAB [Fig. 2(a) and (b)] and compared with activity values obtained when using 150 nmol 10-DAB, may indicate that 50 nmol is a suboptimal substrate concentration in our *in vitro* assay. Inconsistent and statistically insignificant levels of acetylation of 10-deacetyltaxol to yield paclitaxel were observed. The most likely explanation for the inconsistent appearance of paclitaxel in the assays is through the presence of an uncharacterized acetyl CoA:10-deacetyltaxol-10-*O*-acetyltransferase as a contaminant in our enzyme preparation. Throughout the experiments, the controls without added substrate (CS) displayed

consistently higher activity relative to other controls; this may be the result of endogenous 10-DAB present in the protein fraction. Paclitaxel can bind non-specifically to proteins [15] and it is possible that the same property applies to 10-DAB; this would result in some retention of the endogenous taxanes present in the extracted cells even after partial protein purification (i.e. precipitation and anion exchange column fractionation). In fact, the presence of endogenous taxanes associated with the protein extracts was routinely detected by HPLC analysis, in support of the possible explanation above.

The detection of 10-DAB acetyltransferase in leaves and long-term cell suspensions of *Taxus cuspidata* and its partial purification and assay, will allow for detailed investigation of enzyme activity during the growth cycle of cell cultures, as well as the response of the enzyme and the flow of taxane metabolites under normal conditions, as well as to various stimuli (e.g. treatment with jasmonic acid [16]). Further purification and characterization of 10-DAB acetyltransferase will allow better assessment of the exact contribution of this enzyme to the control of taxane metabolism.

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