Coexpression in Yeast of *Taxus* Cytochrome P450 Reductase With Cytochrome P450 Oxygenases Involved in Taxol Biosynthesis

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Abstract: To maximize redox coupling efficiency with recombinant cytochrome P450 hydroxylases from yew (*Taxus*) species installed in yeast for the production of the anticancer drug Taxol, a cDNA encoding NADPH:cytochrome P450 reductase from *T. cuspidata* was isolated. This single-copy gene (2,154 bp encoding a protein of 717 amino acids) resembles more closely other reductases from gymnosperms (~90% similarity) than those from angiosperms (<80% similarity). The recombinant reductase was characterized and compared to other reductases by heterologous expression in insect cells and was shown to support reconstituted taxoid 10β-hydroxylase activity with an efficiency comparable to that of other plant-derived reductases. Coexpression in yeast of the reductase along with *T. cuspidata* taxoid 10β-hydroxylase, which catalyzes an early step of taxoid biosynthesis, demonstrated significant enhancement of hydroxylase activity compared to that supported by the endogenous yeast reductase alone. Functional transgenic coupling of the *Taxus* reductase with a homologous cytochrome P450 taxoid hydroxylase represents an important initial step in reconstructing Taxol biosynthesis in a microbial host.

Keywords: Taxol; paclitaxel; cytochrome P450 reductase; cytochrome P450 taxoid 10β-hydroxylase; cytochrome P450 taxoid 13α-hydroxylase; *Taxus*; yew; *Saccharomyces cerevisiae*; yeast

INTRODUCTION

Taxol (Wall and Wani, 1995) (generic name paclitaxel, Fig. 1) is well established as a potent antineoplastic drug with excellent activity against a range of cancers (Goldspiel, 1997). This taxane diterpenoid (taxoid), derived from yew (*Taxus*) species (Croom, 1995), continues to find wide application in the treatment of additional cancer types (Skeel, 1999), for earlier use in disease intervention (Michaud et al., 2000), and in combination with other chemotherapeutic agents (Brown, 2003). Total syntheses of Taxol have been achieved by several elegant routes (see Kingston et al., 2002, for recent review) but the approach is commercially impractical (Borman, 1994; Xiao et al., 2003). The increased demand for the drug therefore shifted destructive isolation from the original, low-yielding source (the bark of the Pacific yew, *Taxus brevifolia*) to semi-synthetic approaches (see Wuts, 1998, for recent review). These approaches involve synthetic sidechain attachment to the C13-position of more readily available, advanced taxoids, such as 10-deacetylbaccatin III (Fig. 1), that can be isolated in higher yield from the needles of various *Taxus* species as a renewable resource. The supply of Taxol for the foreseeable future will continue to rely on yew species or cell cultures derived therefrom (Cragg et al., 1993; Ketchum and Gibson, 1996; Ketchum et al., 1999; Suffness, 1995), with the production of Taxol or suitable precursors in genetically engineered microorganisms as the pharmaceutically most desirable alternative (Sankawa and Itokawa, 2003; Suffness, 1995). For these reasons, it is important to understand the biosynthesis of Taxol and its underlying molecular genetics, with the view to improving production yields by transgenic means.

The Taxol biosynthetic pathway is considered to require 19 enzymatic steps from the universal diterpenoid precursor geranylgeranyl diphosphate (Jennewein and Croteau, 2001; Walker and Croteau, 2001) which is cyclized, in the committed step, to taxa-4(5),11(12)-diene (Koepp et al., 1995). This parental olefin (Fig. 1) is then functionalized by a series of eight cytochrome P450-mediated oxygenations and two CoA-dependent acylations, and undergoes oxidation at C9 and ring expansion to the oxetane function en route to

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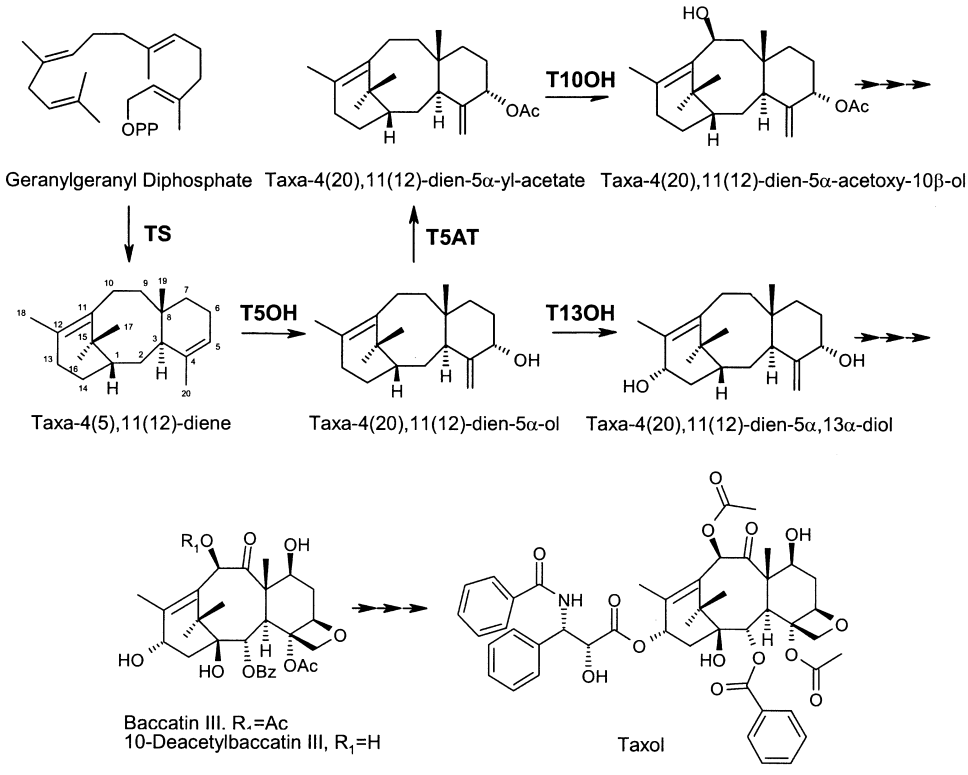

Figure 1. Outline of the Taxol biosynthetic pathway from primary metabolism. The enzymes indicated are taxadiene synthase (TS), taxadiene 5α-hydroxylase (T5OH), taxadienol 5-*O*-acetyltransferase (T5AT), taxoid 10β-hydroxylase (T10OH), and taxoid 13α-hydroxylase (T13OH). Multiple arrows indicate several biosynthetic steps. Ac, acetate; Bz, benzoate. OPP is the diphosphate moiety.

10-deacetyl baccatin III, to which the C10 acetate group is added and the sidechain at C13 is appended (in five additional steps) to afford Taxol. Thus, over half of the reactions required to produce the intermediates 10-deacetyl baccatin III (13 steps) and baccatin III (14 steps) are catalyzed by cytochrome P450 enzymes (eight oxygenations).

The proposed order of these hydroxylations on the taxane core, based on an assessment of the oxygenation patterns of the naturally occurring taxoids (Floss and Mocek, 1995) and cell-free biochemical studies on early pathway steps (Hefner et al., 1996; Lovy Wheeler et al., 2001), is thought to begin with C5 and C10, then C13, C2, and C9 (exact order uncertain), followed by C7, and finally C1. A family of sequence-related cytochrome P450 cDNA clones, isolated by differential display of mRNA-reverse transcription-polymerase chain reaction (DD-RT-PCR) (Schoendorf et al., 2001) and homology-based cloning (Jennewein et al., 2004a), and from an expressed sequence tagged (EST) project (Jennewein et al., 2004b) using induced *Taxus* cell cultures as the source (Ketchum et al., 1999), has yielded genes encoding the taxoid 2α-, 5α-, 7β-, 10β-, and 13α-hydroxylases responsible for these pathway steps (Chau and Croteau, 2004; Chau et al., 2004; Jennewein et al., 2001, 2004a; Schoendorf et al., 2001). The biochemical function of these genes was confirmed by expression in *Saccharomyces cerevisiae* WAT11 cells (Pompon et al., 1996), which coexpress a galactose-inducible NADPH:cytochrome P450 reductase from *Arabidopsis* (Mizutani and Ohta, 1998; Urban et al., 1997) for efficient electron transfer to the recombinant cytochrome, or by expression using the baculovirus-*Spodoptera* system, which is efficient for plant genes (Kraus and Kutchan, 1995; Kutchan et al., 1994) but which relies for catalytic activity on the endogenous insect cell reductase (Gonzales et al., 1991) unless supplemented with a coexpressed alternate reductase (Lee et al., 1996). Thus, in both expression systems, redox coupling to the cytochrome P450 hydroxylase (Peterson and Prough, 1986) is accomplished with a heterologous reductase. The source of the reductase can influence the efficiency of the coupled reaction mediated by cytochrome P450s of plant origin (Pompon et al., 1996; Ponnamperuma and Croteau, 1996) and may even influence the stereochemistry of the reaction (Kraus and Kutchan, 1995). For these reasons, it was of interest to clone a *Taxus* NADPH:cytochrome P450 reductase, examine the coupling efficiency and properties of the recombinant enzyme, and to then deploy the homologous reductase with *Taxus* hydroxylases to maximize redox compatibility (Truan et al., 1993) in preliminary studies directed to engineering taxoid biosynthesis in yeast. In this article we describe the isolation and characterization of the reductase from *T. cuspidata* and its use for coexpression in yeast with the homologous taxoid 10β-hydroxylase and 13α-hydroxylase, the first two taxoid biosynthetic genes of this type to be isolated and defined (Jennewein et al., 2001; Schoendorf et al., 2001).

MATERIALS AND METHODS

Substrates and Reagents

The preparations of [20-^{3}H]taxa-4(20),11(12)-dien-5α-ol and [20-^{3}H]taxa-4(20),11(12)-dien-5α-yl acetate (both at 2 Ci/mol) have been described (Lovy Wheeler et al., 2001; Rubenstein et al., 2000). Rat liver cytochrome b_5 and mung bean NADPH:cytochrome P450 reductase were gifts from R.W. Estabrook (Univ. Texas SW Med. Ctr., Dallas, TX). The commercial sources of other biochemicals and molecular biologicals are indicated in the text and were used according to the manufacturer's instructions.

Cloning of Cytochrome P450 Reductase

Sequence alignment of plant-derived NADPH:cytochrome P450 reductases in the GenBank database revealed several regions of near identity, thereby allowing a straightforward homology-based approach to acquiring a cDNA encoding this reductase from *T. cuspidata*. A degenerate forward primer corresponding to 5′-GMKTTYCCDTCWGCHAA-RCCHCCNCTHGGKG-3′ and a degenerate reverse primer corresponding to 5′-TYACCADACRTCW-CKDAGRTA-WCKTCC-3′, designed to two highly conserved regions, were synthesized and used to amplify a fragment from an induced *T. cuspidata* suspension cell λZAP-II cDNA library (Schoendorf et al., 2001) as template. The amplicon obtained showed good homology to cytochrome P450 reductase sequences, and so was labeled and employed to screen the same library (2×10^5 pfu) from which 16 partial (5′-truncated) clones were obtained. By employing a *T. cuspidata*-induced cell RACE library, constructed using a ClonTech (Palo Alto, CA) Marathon RACE library construction kit, the missing 5′-sequence was readily obtained with which the full-length gene was acquired by PCR. *T. cuspidata* suspension cells used as the source of mRNA were induced for Taxol production with methyl jasmonate (Ketchum et al., 1999).

Constructs and Heterologous Expression

For functional expression and characterization of the newly acquired *T. cuspidata* cytochrome P450 reductase, and for coexpression with *T. cuspidata* cytochrome P450 taxoid 10β-hydroxylase (Schoendorf et al., 2001), the baculovirus-*Spodoptera fugiperda* (Sf9) system was used. For construction of the recombinant baculoviruses, containing either the reductase or the hydroxylase, the corresponding open reading frames (ORFs) were amplified by PCR using *Pfu* DNA polymerase and gene-specific primers containing appropriate restriction sites immediately upstream of the starting ATG and downstream of the stop codon. The amplicons so obtained by standard PCR procedures were gel-purified, subcloned first into pCR-Blunt vectors (Invitrogen, La Jolla, CA), and the ORFs then excised, using the added restriction sites, and ligated into similarly restricted pFastBac vectors (Life Technologies, Bethesda, MD). The orientation and sequence of these reductase and hydroxylase clones were confirmed and the pFastBac constructs were then used for the preparation of the corresponding recombinant Bacmid DNA by transformation of *E. coli* strain DH10Bac (Life Technologies). This protocol, as well as the transfection of *S. fugiperda* Sf9 insect cells, were carried out according to the manufacturer's instructions (Life Technologies). For control experiments, construction was carried out identically, except that the recombinant baculovirus contained a β-glucuronidase gene instead of the cytochrome P450 reductase or hydroxylase gene. Detailed procedures for the propagation of Sf9 cells, protocols for expression (and coexpression), and methods for cell harvest have been described (Jennewein et al., 2001).

Several reductase proteins with different truncations of the presumptive N-terminal membrane insertion sequence were expressed in yeast as fusions with a C-terminal HA epitope-tag. These constructs were amplified with the full-length cDNA as template and the ClonTech Advantage-HF2 PCR kit using upstream primers encoding a *Bam*HI site and corresponding to the truncated sequence: TCPR1-N (5′-CCAGGATCCATGCAGGCTAATTCC-3′) encodes MQANS (aa 1–5); TCPR1-1N (5′-CCAGGATCCATGACC GGCTCGGCTTTG-3′) encodes MTGSAL (aa 41–46); TCPR1-2N (5′-CCAGGATCCATGTGGAGGAGGGGAG-GA-TCGGATAC-3′) encodes MWRRGGSDT (aa 74–81); the common downstream primer TR2 (5′-CGCGTCGACT-CAAGCGTAATCTGGAACATCGTATGGGTACCATA-TATCTCGTAAGTATC) incorporates an *Sal*I site, encodes RYLRDIW (aa 711–717), followed by YPYDVPDYA (the HA epitope-tag sequence) and the stop codon. The following PCR conditions were employed: 5 min at 95°C, then 30 cycles for 40 sec at 95°C, 40 sec at 55°C, and extension for 2 min at 72°C, with final extension for 5 min at 72°C. The resulting amplicons were digested with *Bam*HI and *Sal*I, gel-purified, ligated into the corresponding sites of p425GAL1 or p425GPD (both from American Type Culture Collection, Rockville, MD), and individually transformed into *E. coli* TOP10 competent cells (Invitrogen) for screening using the cracking gel technique and plasmid preparation using the mini-prep kit (Promega, Madison, WI).

For coexpression studies using the pESC-Trp yeast dual expression vector (Stratagene, La Jolla, CA), the cytochrome P450 taxoid 10β-hydroxylase (Schoendorf et al., 2001) and the cytochrome P450 taxoid 13α-hydroxylase (Jennewein et al., 2001) were each amplified by PCR using *Pfu* DNA polymerase and gene-specific primers to introduce 5′-*Not*I and 3′-*Cla*I restriction sites in the former. In the latter case, 5′-*Bam*HI and 3′-*Sal*I restriction sites were introduced in addition to a C-terminal VSVG epitope-tag (YTDIEMNRLGK). The resulting sequence-verified amplicon for the 10β-hydroxylase was digested with *Not*I/*Cla*I and directionally inserted into the corresponding site behind the GAL10 promoter in-frame with the C-terminal FLAG epitope (DYKDDDDK). The resulting sequence-verified amplicon for the 13α-hydroxylase was digested

with *Bam*HI/*Sal*I and inserted into the corresponding site behind the GAL1 promoter in-frame with the VSVG epitope in the proper orientation.

Procedures used for the maintenance, transformation, and induction of *S. cerevisiae* were those described in the Invitrogen manual. *S. cerevisiae* YPH499 competent cells (Stratagene) were prepared using the EasyComp Transformation kit (Invitrogen) and were transformed/cotransformed with 1 μg of the relevant plasmid(s) according to the manufacturer's protocol. Transformed cells were selected on SD-Trp dropout plates and one or more colonies were used to inoculate SD-Trp dropout liquid medium for overnight culture. The cells were then harvested by centrifugation, suspended in galactose-Trp dropout medium to give A_{600} ~0.4, and the induced cultures grown at 37°C with vigorous shaking. At various times postinduction, aliquots of the suspended cells were taken for immunoblotting and enzyme assay.

Immunoblotting and Enzyme Assays

For instances in which the subcellular distribution of the recombinant reductase was uncertain (i.e., the expression of N-terminally truncated forms), crude cell lysates (prepared by disruption of the cell pellet by vortex mixing with glass beads for 5 × 30 sec at 4°C and low-speed centrifugation) were employed for immunoblotting and activity assay. Otherwise, established protocols for microsome preparation from insect cells (Jennewein et al., 2001, 2004b) and yeast (Pompon et al., 1996) were employed. Membrane content of cytochrome P450 was determined by CO-difference spectrometry (Omura and Sato, 1964) as described in detail elsewhere (Schenkman and Jansson, 1998). Protein content was determined by the Bradford (1976) method or using the Coomassie Plus kit (Pierce, Rockford, IL) with bovine serum albumin as standard. Established protocols for membrane solubilization and reconstitution (i.e., with various reductases and/or cytochrome b_5) were employed (Haudenschild et al., 2000; Shimada and Yamazaki, 1998); the solubilization efficiency using Emulgen 911 (Kao Chemical, Tokyo) as detergent was about 50%.

Procedures for immunoblotting of fusion proteins bearing an appended C-terminal tag have been described (Jennewein et al., 2003, 2004a). Primary antibodies were mouse anti-HA, mouse anti-FLAG, and mouse anti-VSVG (all from Sigma, St. Louis, MO), depending on the target. Anti-peptide antibodies were generated in rabbits by the contractor (Genemed, South San Francisco, CA) against two regions of the *T. cuspidata* reductase, the TDR1A sequence corresponding to SDTQKPAVRPTPLVKEED (aa 79–96 with an appended C) and the TDR2B sequence corresponding to SVYEETHALKQNGQAVYD (aa 296–313 with an appended C). Following SDS-PAGE with size markers and transfer in Tris-glycine buffer to PVDF membranes using a Bio-Rad (Hercules, CA) transfer apparatus, the membranes were incubated with SuperBlock blocking buffer (Pierce) and the appropriate primary antibody. The secondary antibody was either sheep anti-mouse or antirabbit IgG, as appropriate, conjugated with horseradish peroxidase (Sigma). Membranes were washed with Tris-buffered saline, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 min, then exposed to X-ray film.

NADPH:cytochrome P450 reductase activity was determined via the (nonphysiological) reduction of cytochrome c, which was measured spectrophotometrically at 550 nm (Gupta and Porter, 2001; Lord, 1983) employing a molar absorption coefficient of 21 cm^{-1} mM^{-1} (Van Gelder and Slater, 1962). The standard assay contained 6 μg/ml microsomal protein, 0.2 mM oxidized bovine cytochrome c (Sigma, USA) and 2.5 μM each of FMN and FAD in 10 mM potassium phosphate buffer (pH 7.7) with 0.1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol. The reaction was initiated by addition of freshly prepared NADPH (Sigma) to a final concentration of 50 μM; assays were run in triplicate and the standard errors (SE) were within ± 11% of the mean in all cases. Following the establishment of linear reaction conditions in protein concentration and time, the pH optimum for the reductase was determined in 0.25 M Tris-Cl (pH 7.0–9.0) and 0.25 M potassium phosphate (pH 5.5–8.5) buffers over intervals of 0.5 pH units. The *K*m value for NADPH was determined under optimum assay conditions at concentrations ranging from 1–100 μM. The SPSS (Chicago, IL) Sigmaplot Enzyme Kinetics 1.10 software package was used with the Michaelis-Menten method (R^2 = 0.98 for the line of best fit), and the data reported are the means ± SE of triplicate analyses. Purified recombinant human NADPH:cytochrome P450 reductase (PanVera, Invitrogen) was used as positive control and to calibrate the assay.

The assays for cytochrome P450 taxoid 10β-hydroxylase (with [20-^{3}H]taxadien-5α-yl acetate as substrate) and for cytochrome P450 taxoid 13α-hydroxylase (with [20-^{3}H] taxadien-5α-ol as substrate) have been described in detail elsewhere (Jennewein and Croteau, 2001; Lovy Wheeler et al., 2001; Schoendorf et al., 2001). Following incubation of these microsomal preparations in the presence of NADPH and O_2 in the dark, the radiolabeled product and residual substrate were extracted and separated by reversed phase radio-HPLC, with quantification based on the labeled substrate of defined specific activity; assays were run in triplicate and the means ± SE are reported.

RESULTS

Isolation of a *T. cuspidata* Cytochrome P450 Reductase

Based on multiple sequence alignment of the dozen defined plant NADPH:cytochrome P450 reductases in GenBank, two highly conserved regions were identified to which degenerate deoxyoligonucleotide primers were designed. Using these primers in an RT-PCR reaction, with source

mRNA obtained from *T. cuspidata* cells induced with methyl jasmonate for increased taxoid production (Ketchum et al., 1999; Schoendorf et al., 2001), led to the isolation of a predicted 600-bp cDNA fragment that showed good homology to other cytochrome P450 reductases of plant origin. This cDNA fragment was then employed as a probe in screening the *T. cuspidata* cDNA library (Schoendorf et al., 2001). The screening of $\sim 2 \times 10^5$ cDNA clones yielded 16 apparently identical partial sequences, all of which were truncated at the 5′-terminus. By using a *T. cuspidata* rapid amplification of cDNA ends (RACE) library, the 5′-terminal sequence was obtained and the full-length clone was acquired by PCR (GenBank accession no. AY571340); this clone of 2941 bp contains an ORF of 2154 bp starting at position 98 and codes for a 717-aa protein.

The deduced sequence showed, by BLAST searching (Pearson and Lipman, 1988) against sequences in the public domain, good homology to other plant NADPH: cytochrome P450 reductases, with 64–69% identity and 75–80% similarity scores compared to those of angiosperms (both monocots and eudicots), and ~80% identity and ~90% similarity scores compared to those of the very few gymnosperms (*Pseudotsuga menziesii* (Douglas fir) and a partial sequence from *Picea abies* (Norway spruce)) in the database. The nucleotide sequence of the *T. cuspidata* reductase ORF is essentially identical to that of the reductase previously acquired from *T. chinensis* (Jennewein, 2000) in differing in only 10 nucleotide positions (of 2154 nt), four of which are silent and six of which lead to conservative amino acid substitutions. The above library screen yielded only one reductase sequence, which is consistent with the results of a recent EST project, using the same induced *T. cuspidata* cells as source, that afforded only this one reductase at 0.6‰ abundance (Jennewein et al., 2004b). Genomic Southern blot analysis (data not shown) confirmed the presence of the putative *T. cuspidata* NADPH:cytochrome P450 reductase as a single-copy gene.

Expression of Cytochrome P450 Reductase in Insect Cells

For functional expression of the *Taxus* cytochrome P450 reductase, the baculovirus-*Spodoptera fugiperda* (Sf9) system was chosen. This eukaryotic system allows reasonably efficient expression of plant membranous recombinant proteins and allows convenient microsome preparation (Kraus and Kutchan, 1995; Kutchan et al., 1994). Furthermore, if the *Taxus* reductase could be successfully expressed in Sf9 cells, it could provide an alternative, compatible coexpression system for examining cytochrome P450 taxoid oxygenase clones that do not express well, or are unstable, in the WAT11 yeast host (Jennewein et al., 2001). By using the assay based on NADPH-dependent reduction of cytochrome c (Lord, 1983), microsomes of control Sf9 cells (which express β-glucuronidase) were shown to contain modest levels of cytochrome P450 reductase activity (12.5 ± 2.5 milliunits (mU)/mg protein). Heterologous expression of the *T. cuspidata* cytochrome P450 reductase in these cells led to an ~15-fold increase in reductase activity over endogenous levels (i.e., >200 mU/mg microsomal protein). The recombinant *T. cuspidata* reductase displayed a pH optimum near pH 7.5 in 0.25 M Tris buffer, with half-maximum velocity at pH 7.0 and pH 8.5. The *Km* value for NADPH was determined to be 17.8 ± 2.9 μM, again using the assay based on the reduction of cytochrome c.

Coexpression of NADPH:Cytochrome P450 Reductase and Cytochrome P450 Taxoid 10β-Hydroxylase in Insect Cells

To examine the ability of the cloned NADPH:cytochrome P450 reductase to support cytochrome P450 hydroxylase activity in vitro, the *T. cuspidata* reductase was coexpressed with the previously described *T. cuspidata* cytochrome P450 taxoid 10β-hydroxylase (GenBank accession no. AF318211) (Schoendorf et al., 2001) in Sf9 cells. By coexpression of the two, 250 pmol of the heterologously expressed cytochrome P450 (judged by the CO-difference spectrum) and about 200 mU of reductase activity (judged by cytochrome c reduction) were obtained (per mg protein) in the derived microsomes. Functional capability of the *T. cuspidata* reductase in electron transfer from NADPH to the cytochrome P450 oxygenase was determined by standard assay of the microsomal taxoid 10β-hydroxylase activity with its natural substrate taxadien-5α-yl acetate (Schoendorf et al., 2001). Transformation of Sf9 cells with both the cytochrome P450 and the reductase led to an increase in taxoid 10β-hydroxylase activity by 3-fold over that of cells transformed with the cytochrome P450 hydroxylase alone and which relied solely on the endogenous insect cell reductase for function (see Table I).

Influence of Reductase Source and Cytochrome b_5 on Taxoid 10β-Hydroxylase

To evaluate the influence of different NADPH:cytochrome P450 reductases on taxoid hydroxylase activity, microsomal preparations derived from insect cells expressing only the taxoid 10β-hydroxylase were separately reconstituted by a

Table I. Effects of cytochrome P450 reductase and cytochrome b_5 on taxoid 10β-hydroxylase.

Cyt P450 reductase (mU)	Cyt 450 taxoid 10β-hydroxylase (pmol)	Cty b_5 (pmol)	Hydroxylase turnover (h^{-1})
—	30	—	65 ± 15
25	30	—	210 ± 18
—	30	6	105 ± 20
25	30	6	340 ± 20

The recombinant reductase and cytochrome P450 were coexpressed in Sf9 cells as indicated, and cytochrome b_5 was reconstituted into the resulting insect microsomal membranes. No entry indicates endogenous levels in the insect cells. Turnover is expressed as nmol product/nmol cytochrome P450/h ± SE.

standard protocol (Haudenschild et al., 2000; Shimada and Yamazaki, 1998) with the reductases from *T. cuspidata*, spearmint (Ponnamperuma and Croteau, 1996), mung bean (Shet et al., 1993), and rabbit (Sigma) at 1:1 ratio (mU reductase:pmol cytochrome P450). The rabbit reductase was least efficient (by ~60%) in supporting the hydroxylation reaction but insignificant differences were observed with the three reductases of plant origin (data not shown), indicating that, in spite of the evolutionary distance between the gymnosperm cytochrome P450 and the angiosperm reductases (and the sequence differences between the *T. cuspidata* reductase and the others), the source of plant-derived reductase was of little consequence, at least for the taxoid 10β-hydroxylase.

Cytochrome b_5 has been shown to exert various influences on cytochrome P450 catalytic systems, with enhancement, no effect, and even inhibition having been observed, and no clear consensus has emerged as to whether the influence of cytochrome b_5 relates to electron transfer capability or to only physical association with the particular cytochrome P450 (Auchus et al., 1998; Waskell et al., 1991; Yamazaki et al., 1996). To examine the influence of cytochrome b_5 on the taxoid 10β-hydroxylase, microsomal preparations from insect cells expressing only the cytochrome P450 taxoid 10β-hydroxylase alone, or coexpressing the hydroxylase with the cytochrome P450 reductase, were reconstituted as before but with purified rat cytochrome b_5. At near optimum reductase to cytochrome P450 ratio (~1 mU:1 pmol), saturation with cytochrome b_5 was achieved in the 5–6 pmol range with a slight decrease in effect at 30 pmol (data not shown). Cytochrome b_5 led to a relative 60% increase in taxoid 10β-hydroxylase activity, either in the absence (i.e., endogenous levels) or presence of recombinant *Taxus* cytochrome P450 reductase (3-fold absolute increase in this case), with maximal overall activity observed in the presence of both recombinant reductase and cytochrome b_5 (Table I).

Expression of Cytochrome P450 Reductase in Yeast

With cloning of the *Taxus* cytochrome P450 reductase completed and preliminary characterization of the recombinant enzyme in *Spodoptera* microsomal preparations in progress, attention was turned to a parallel effort to install functional taxoid hydroxylating systems in yeast as the ultimate drug-production platform. For preliminary studies, the full-length reductase with a C-terminally appended HA-tag, and under the control of the inducible GAL1 promoter, was prepared for episomal expression. Two independently prepared constructs (p425GAL1-TCPR*1 and p425GAL1-TCPR*2) and the empty vector control (p425GAL1) were transformed separately into YPH499 yeast cells, and, following growth and induction, the cells were harvested at various times for microsomal protein isolation and immunoblotting using commercially available monoclonal antibodies directed against the appended epitope (Fig. 2). The reductase (of the expected 86 kDa size) was detected at

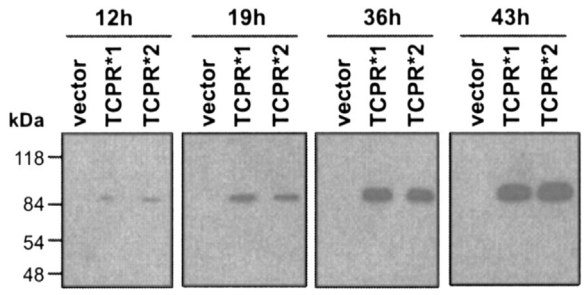

Figure 2. Immunoblotting of *T. cuspidata* NADPH:cytochrome P450 reductase expressed in yeast as a function of induction time. YPH499 cells were transformed with p425GAL1-TCPR*1 or p425GAL1-TCPR*2 (two independent representatives of the full-length reductase), or with p425GAL1 (empty vector control). Yeast cells were collected at the indicated times postinduction, the microsomes were prepared and extracted, and equal aliquots were subject to SDS-PAGE, blotting, and detection with monoclonal antibodies directed against the C-terminal HA-epitope. The 12-h blot was exposed to film for 10 min; the remainder for 30 sec.

12 h postinduction for both positive constructs, but not for the negative control, and the level of this enzyme (as a function of total microsomal protein) increased continuously for at least 43 h.

For this yeast system, in which the coexpression of several Taxol biosynthetic enzymes is contemplated, specific antibody probes for each pathway catalyst would be most desirable. Therefore, polyclonal antibodies were generated against two reductase peptides, TDR1A (aa 79–96), which is a highly hydrophilic region immediately following the presumptive membrane insertion sequence, and TDR2B (aa 296–313), which corresponds to an internal segment that also shares little homology with other P450 reductases, yeast proteins, or other Taxol pathway enzymes. The anti-TDR2B antibodies were of limited utility because of high background; however, the anti-TDR1A polyclonal antibodies were specific, showed negligible background, and did not crossreact with other pathway enzymes tested, including geranylgeranyl diphosphate synthase (Hefner et al., 1998) and taxadiene synthase (Wildung and Croteau, 1996) (Fig. 3), as well as taxadienol 5-*O*-acetyltransferase (Walker et al., 2000) and cytochrome P450 taxoid 10β-hydroxylase (Schoendorf et al., 2001) (data not shown).

Cytochrome P450 reductases are thought to bind to the endoplasmic reticulum via the N-terminal hydrophobic membrane integration region (~aa 1–75) (Durst and Nelson, 1995; Masters and Okita, 1982), but previous studies (Venkateswarlu et al., 1998) have indicated that this N-terminal membrane insertion sequence may or may not be required for reductase function. To evaluate the role of the N-terminus, the full-length reductase (i.e., aa 1–717) was compared to partially deleted forms (aa 41–717 and aa 56–717) and to a version from which the entire insertion sequence had been deleted (i.e., 74–717), either with or without the C-terminal HA-tag. Each construct was expressed under control of the GAL1 promoter, and crude extracts were prepared from harvested cells at various times following induction and were evaluated by immunoblotting

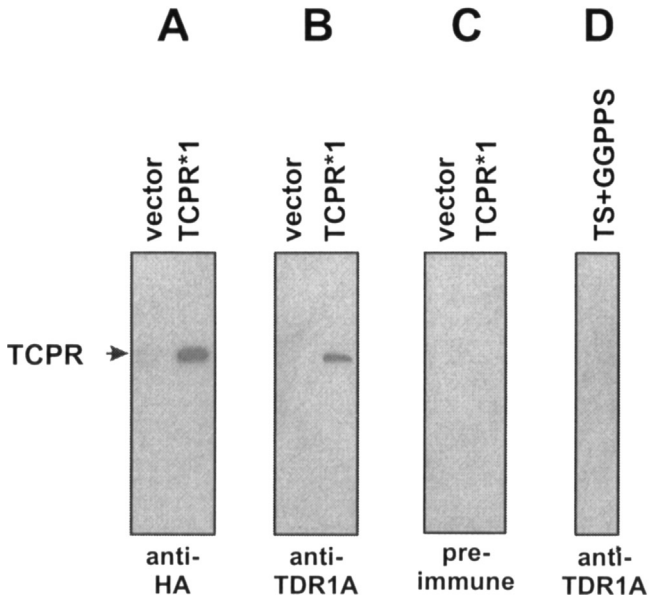

Figure 3. Immunoblotting of *T. cuspidata* NADPH:cytochrome P450 reductase expressed in yeast using the anti-TDR1A (peptide specific) antibodies. TCPR*1 and the empty vector control (vector) were expressed and prepared as described in Figure 2 (36 h postinduction). Lane A illustrates detection with antibodies directed against the C-terminal HA-epitope (anti-HA). Lanes B and C illustrate detection of the same sample with the reductase peptide-specific antibodies (anti-TDR1A) and the lack thereof with the corresponding preimmune control. Lane D illustrates the failure of anti-TDR1A to crossreact with two other recombinant pathway enzymes, geranylgeranyl diphosphate synthase (GGPPS) and taxadiene synthase (TS), expressed in yeast from pESC-Ura. The anti-TDR1A antibodies also failed to detect taxadienol 5α-*O*-acetyltransferase and cytochrome P450 taxoid 10β-hydroxylase in experiments similarly performed.

with the anti-TDR1A-specific antibodies (the sizes of the expressed proteins were as predicted; data not shown) and by enzyme assay (cytochrome c reduction per mg crude protein) (Fig. 4). Interestingly, the highest expression level (by immunoblotting) and highest activity (by reductase assay) at all time points were observed for the reductase from which both N-terminal insertion sequence and C-terminal tag had been deleted (about 8-fold higher activity levels than empty vector control at 30 h). These results indicated that both the N-terminal membrane insertion sequence and the C-terminal HA-tag adversely influenced the expression of catalytically functional reductase and that (by comparison with the various intermediate truncated and tag-deleted forms) the N-terminus exerted the greatest negative influence (by about 3-fold compared to less than 2-fold for the C-terminal tag). This experiment was repeated with the same set of constructs but under the control of the GPD (constitutive) promoter (Fig. 4) and, although overall expression levels were reduced by about half, the conclusions were the same.

Coexpression of Reductase With Taxoid Hydroxylases in Yeast

In order to reconstitute functional taxoid hydroxylating systems in situ in the yeast host, the variously modified cytochrome P450 reductases were coexpressed with two early taxoid pathway cytochrome P450 hydroxylases using the Gal-inducible yeast dual expression vector pESC-Trp (Stratagene). The taxoid 10β-hydroxylase (Schoendorf et al., 2001) was inserted behind the GAL10 promoter in-frame with the vector encoded C-terminal FLAG epitope, and the taxoid 13α-hydroxylase (Jennewein et al., 2001) was similarly inserted in the same vector behind the GAL1 promoter with the addition of a C-terminal VSVG epitope; C-terminal tagging is without significant influence on

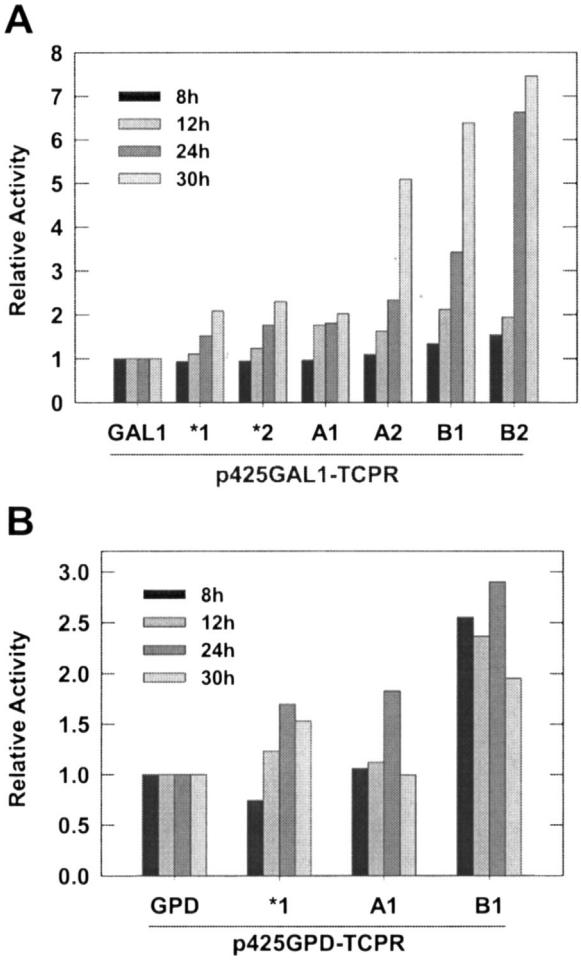

Figure 4. Expression and assay of full-length and truncated forms of *T. cuspidata* NADPH:cytochrome P450 reductase. Crude extracts of YPH499 cells transformed with various reductase (TCPR) constructs under control of the GAL1 promoter (**A**) or constitutive GPD promoter (**B**) were prepared at the indicated times postinduction and equal aliquots were assayed in triplicate for (cytochrome c) reductase activity (averages are reported and the standard errors were within ± 11% of the means). The constructs are the empty vector (endogenous reductase) control (GAL1 and GPD), two independently prepared full-length forms (aa 1–717) with C-terminal HA-tag (*1), and without the tag (*2), the partially truncated form (aa 41–717) with C-terminal HA-tag (A1), the partially truncated form (aa 56–717) without the HA-tag (A2), the fully truncated form (aa 74–717) with C-terminal HA-tag (B1), and the fully truncated form without the HA-tag (B2). Immunoblotting of the same extracts with the anti-TDR1A peptide-specific antibodies showed expression levels to roughly correspond to activity levels. Expression of the reductase (all forms) from p425GPD was roughly half as efficient as that from p425GAL1.

these cytochrome P450 hydroxylases and permits ready immunodetection of the recombinant enzymes. The corresponding yeast strains were generated by independent cotransformation with the reductase/hydroxylase constructs and, following induction, the transformed yeast cells were harvested and microsomes were prepared for immunoblotting and enzyme assay. Both the taxoid 10β-hydroxylase and the 13α-hydroxylase were readily detected in the microsomes, as expected, using the appropriate antibodies for the respective epitope tags (Fig. 5). Notably, all of the reductase forms were found to reside in the microsomal fraction by immunoblotting with anti-TDR1A (Fig. 5), regardless of whether they contained the full N-terminus, or the partially or fully deleted membrane integration sequence. These results are consistent with previous suggestions (Estabrook et al., 1996; Venkateswarlu et al., 1998) that the reductase may be recruited to the endoplasmic reticulum membrane by association with their cytochrome P450 partners in the absence of the membrane insertion sequence. However, it should be noted here that we did not search for soluble forms of the truncated reductase in the absence of coexpressed cytochrome P450, so that no firm conclusions can be drawn regarding recruitment of the reductase or the role of the presumptive membrane insertion sequence. The activity levels of the various microsomal reductase forms (by cytochrome c reduction on a total microsomal protein basis) very roughly paralleled the observed expression level (by immunoblotting on a total microsomal protein basis) with the B2 version of the reductase (both N-terminal insertion sequence and C-terminal tag deleted) showing the highest level of activity (Fig. 5).

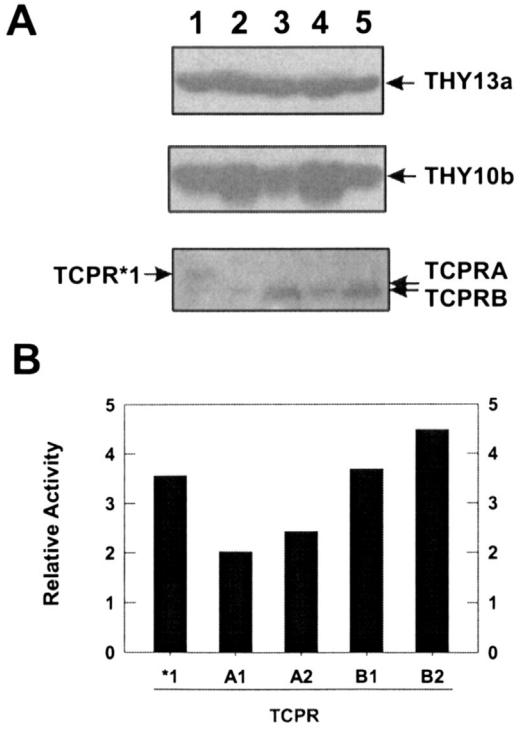

Figure 5. Expression and assay of full-length and truncated forms of the reductase in the presence of two taxoid hydroxylases. Microsome preparations from YPH499 cells transformed with the various reductase (TCPR) constructs (described in Fig. 4), and with cytochrome P450 taxoid 13α-hydroxylase (THY13a) and taxoid 10β-hydroxylase (THY10b), all under control of the GAL1/GAL10 promoters, were obtained 24 h post-induction, and 10 μg of microsomal protein was subjected to immunoblotting (**A**) and assay (**B**) in triplicate for (cytochrome c) reductase activity (averages are reported and the standard errors were within ± 9% of the means). Monoclonal antibodies used to detect the different recombinant C-terminally tagged proteins were anti-FLAG (for THY10b), anti-VSVG (for THY13a), and anti-TDR1A (for the various reductase forms). Lanes 1–5 in **A** correspond to the TCPR constructs *1–B2 in **B**.

To assess the ability of the homologous reductase to couple to and support the two coexpressed cytochrome P450 hydroxylases, assays with these preparations were conducted for taxoid hydroxylation using microsomes that harbored the full-length reductase (designated ∗1) and the N-terminally and C-terminally deleted form (designated B2), as well as those that harbored only the endogenous yeast reductase (i.e., designated GAL1 of Fig. 4). Thus, by standard assay in the presence of 50 μM NADPH and O_2 in the dark, the conversion of [20-^{3}H]taxa-4(20),11(12)-dien-5α-yl acetate to taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol by the taxoid 10β-hydroxylase (Schoendorf et al., 2001) and of [20-^{3}H]taxa-4(20),11(12)-dien-5α-ol to taxa-4(20), 11(12)-dien-5α,13α-diol by the taxoid 13α-hydroxylase (Jennewein et al., 2001) (see Fig. 1) were measured by extractive product isolation and radio-HPLC (Table II). Both versions of the reductase (that differed in activity levels by less than 30%) were capable of supporting the taxoid 10β-hydroxylase reaction at levels approximately seven times that of the endogenous yeast reductase, indicating that the recombinant *T. cuspidata* reductase could functionally couple to the recombinant cytochrome P450 hydroxylase in the microbial host. In the case of the taxoid 13α-hydroxylase, activity levels were quite low (in spite of the immunoblotting data indicative of successful over-

Table II. Effect of coexpression of two cytochrome P450 reductase forms on two taxoid hydroxylases.

	Rate of hydroxylation	
Source of reductase	10β-Hydroxylase (nmol/h/mg protein)	13α-Hydroxylase (nmol/h/mg protein)
Yeast (endogenous)	23.4 ± 4.4	16.1 ± 3.8
Taxus (full length)	164 ± 18	18.4 ± 3.7
Taxus (truncated)	158 ± 17	17.7 ± 3.8

Rates (±SE) were measured in yeast microsomes (total protein basis) harboring both recombinant taxoid hydroxylase and the coexpressed *T. cuspidata* reductase bearing both N-terminal membrane anchor and C-terminal HA-tag [*Taxus* (full length)], or the coexpressed *T. cuspidata* reductase from which both N-terminal anchor and C-terminal epitope tag were deleted [*Taxus* (truncated)], or the coexpressed empty vector control [Yeast (endogenous)]. Activity levels of the full length (*1) and truncated (B2) forms of the reductase differed by less than 30% (see Fig. 5) and were in excess of apparent saturation. Empty vector controls (devoid of the hydroxylase inserts) showed both 10β- and 13α-hydroxylase activities to be negligible in microsomes of the yeast host (<0.3 nmol/h/mg protein).

expression; Fig. 5) and no significant difference was observed in the presence of either form of the recombinant reductase at apparent saturation (Table II). It seems unlikely that the recombinant reductase failed to productively couple with the recombinant 13α-hydroxylase in yeast because productive coupling of the two was previously demonstrated in insect cells (Jennewein et al., 2001). Nor does it seem that the mixed redox environment, resulting from the presence of both the *T. cuspidata* and endogenous yeast reductases, was detrimental because productive coupling of the closely related 10β-hydroxylase was demonstrated under these same conditions. However, the taxoid 13α-hydroxylase has proven to be somewhat difficult to functionally express in yeast (compared to the expression in *Spodoptera* cells [Jennewein et al., 2001] and to the expression of the 10β-hydroxylase in both systems [Schoendorf et al., 2001]) and, of the recombinant taxoid hydroxylases thus far examined, it is the least stable in isolated yeast microsomes (Jennewein et al., 2001). Thus, it seems most likely that the endogenous yeast reductase was sufficient to saturate the limited amount of functional 13α-hydroxylase present in this cell-free system, and that no further gain in activity was possible in the presence of excess recombinant reductase. Feeding studies with the taxadien-5α-ol substrate using the intact transformed yeast might offer an alternative, in vivo means of assessing the coupling of recombinant reductase and the taxoid 13α-hydroxylase, and another approach to comparing the 13α- and 10β-hydroxylase systems.

DISCUSSION

The cDNA encoding the NADPH:cytochrome P450 reductase from *T. cuspidata* resembles in sequence other reductases of plant origin, and is more similar to those from gymnosperms than to those from angiosperms, as might be anticipated from the lineage of the Taxaceae (Florin, 1948; Page, 1990). The reductase, as a single-sequence acquisition, was relatively abundant in the induced *T. cuspidata* cell EST library and was shown to be present in the *Taxus* genome as a single-copy gene. This contrasts with studies on several angiosperm species that have demonstrated the presence of multiple cytochrome P450 reductases, one of which appears to be stress-inducible (Koopmann and Hahlbrock, 1997; Mizutani and Ohta, 1998; Ro et al., 2002).

The recombinant enzyme, expressed in insect cells and assayed with NADPH and the surrogate substrate cytochrome c, is similar to other cytochrome P450 reductases in general properties (Benveniste et al., 1986, 1991; Ponnamperuma and Croteau, 1996; Shet et al., 1993). The *T. cuspidata* reductase was comparable to other plant-derived reductases in supporting functional oxygenation by reconstitution with the *T. cuspidata* taxoid 10β-hydroxylase. Thus, although there are instances in which the nature of the reductase influences cytochrome P450 catalysis (Kraus and Kutchan, 1995; Pompon et al., 1996; Ponnamperuma and Croteau, 1996), in the case of the taxoid 10β-hydroxylase the source of the reductase is of little consequence; there are other examples of this phenomenon (Cabello-Hurtado et al., 1998; Mizutani and Ohta, 1998; Urban et al., 1997). Nevertheless, in transgenic applications, for which a series of different cytochrome P450-mediated oxygenation steps is to be employed, redox compatibility is best ensured by the use of the homologous reductase.

The influence of cytochrome b_5 on cytochrome P450 catalysis can be quite variable, depending on the specific system. In the present instance (taxoid 10β-hydroxylase), a 60% increase in activity was observed by reconstituting rat cytochrome b_5 into insect cell microsomes harboring both *Taxus* reductase and hydroxylase. To more fully assess the in situ influence of cytochrome b_5 will require coexpression of the *Taxus* cytochrome in yeast along with the *Taxus* reductase and several representative cytochrome P450 taxoid hydroxylases. Any potential gain in hydroxylase flux by this approach will need to be balanced against the operational limitations to stacking this additional gene into what will already be a substantial and complex set of genes needed to reach the Taxol precursor baccatin III from primary metabolism.

Coexpression of the *Taxus* reductase with the homologous taxoid 10β-hydroxylase in the microbial host demonstrated successful coupling by the substantial increase in hydroxylase activity compared to that supported by the endogenous yeast reductase alone. Such functional transgenic redox coupling represents an important initial step in reconstructing the biosynthesis of Taxol and its precursors in the yeast host because nearly half of the pathway steps are comprised of cytochrome P450-mediated oxygenation reactions. Successful coupling of the *Taxus* reductase with the homologous taxoid 13α-hydroxylase could not be demonstrated, likely because of the previously noted (Jennewein et al., 2001) instability of this hydroxylase and its resulting low activity recovered in the yeast microsomal system used to assess the hydroxylation reaction; the endogenous yeast reductase appeared sufficient to saturate the limiting amount of functional microsomal hydroxylase that was present in this case. Although it should be possible to evaluate coupling of the reductase and taxoid 13α-hydroxylase by in vivo feeding of the intact transformed yeast with the labeled taxadien 5α-ol substrate, it would be most informative to first install the required pathway steps leading to this intermediate (i.e., taxadiene synthase and taxadien 5α-hydroxylase, as well as the taxadienol 5-*O*-acetyltransferase) in order to additionally determine flux through this and the taxoid 10β-hydroxylation branch of the pathway (see Fig. 1). This effort is under way and will be reported at a later date.

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