CYP725A4 from Yew Catalyzes Complex Structural Rearrangement of Taxa-4(5),11(12)-diene into the Cyclic Ether 5(12)-Oxa-3(11)-cyclo税收ane

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Taxa-4(5),11(12)-diene is the first committed precursor of functionalized taxanes such as paclitaxel, a successful anticancer drug. Biosynthesis of taxanes in yew involves several oxidations, a number of which have been shown to be catalyzed by cytochrome P-450 oxygenases. Hydroxylation of the C-5α of taxa-4(5),11(12)-diene is believed to be the first of these oxidations, and a gene encoding a taxa-4(5),11(12)-diene 5α-hydroxylase (CYP725A4) was recently described (Jennewein, S., Long, R. M., Williams, R. M., and Croteau, R. (2004) Chem. Biol. 11, 379–387). In an attempt to produce the early components of the paclitaxel pathway by a metabolic engineering approach, cDNAs encoding taxa-4(5),11(12)-diene synthase and CYP725A4 were introduced in Nicotiana sylvestris for specific expression in trichome cells. Their co-expression did not lead to the production of the expected 5α-hydroxylaxa-4(20),11(12)-diene. Instead, taxa-4(5),11(12)-diene was quantitatively converted to a novel taxane that was purified and characterized. Its structure was determined by NMR analysis and found to be that of 5(12)-oxa-3(11)-cyclo税收ane (OCT) in which the eight-carbon B-ring from taxa-4(5),11(12)-diene is divided into two fused five-carbon rings. In addition, OCT contains an ether bridge linking C-5 and C-12 from opposite sides of the molecule. OCT was also the sole major product obtained after incubation of taxa-4(5),11(12)-diene with NADPH and microsomes prepared from recombinant yeast expressing CYP725A4. The rearrangement of the taxa-4(5),11(12)-diene ring system is thus mediated by CYP725A4 only and does not rely on additional enzymes or factors present in the plant. The complex structure of OCT led us to propose a reaction mechanism involving a sequence of events so far unknown in P-450 catalysis.

The diterpenoid paclitaxel (marketed as Taxol®) isolated from yew is an effective drug widely used in the treatment of numerous cancers (1). Total chemical synthesis of paclitaxel has been described (2) but is not commercially viable because of very low yield (3). Paclitaxel is currently produced by extraction of yew needles and yew cells, and its analog Taxotere® is produced by semi-synthesis from the natural product 10-deacetyl-baccatin III, which is also extracted from yew needles (4, 5). Development of alternative systems for higher yield and simpler process was however pointed out as a strategic priority (6). Yew tree-derived cultures (7) and yeast (8) or plant (9–11) engineering have recently been proposed as promising bio-factories for producing taxane ingredients. In this context, it is essential to understand the biosynthesis of paclitaxel (12).

The isolation and characterization of naturally occurring taxoids have led to the hypothesis that at least 19 biochemical steps are required for the biosynthesis of paclitaxel starting from geranylgeranylpyrophosphate (GGPP), the universal precursor of diterpenoids (13, 14). Although the pathway is not yet understood overall, several important steps have been elucidated in recent years (14). The first committed step involves cyclization of GGPP to taxa-4(5),11(12)-diene by taxa-4(5),11(12)-diene synthsese (TS). The TS enzyme has been characterized, and its cDNA has been cloned (15). Cyclization is thought to be followed by α-hydroxylation of taxa-4(5),11(12)-diene at the C-5 position, with allylic migration of the 4(5) double bond to the 4(20) position, thus leading to 5α-hydroxytaxa-4(20),11(12)-diene (16). This compound is also presumed to be the second intermediate of the paclitaxel pathway, because feeding Taxus brevifolia stem discs with labeled 5α-hydroxytaxa-4(20),11(12)-diene led to the labeling of advanced taxoids such as 10-deacetylbaccatin III, cephalomannine, and paclitaxel (17). The cDNA coding for a taxa-4(5),11(12)-diene 5α-hydroxylase (T5αH) (GenBankTM accession number AY289209;1 called CYP725A4 in P-450 oxygenase nomenclature) was cloned and functionally expressed in yeast and S9 insect cells (16). The catalytic efficacy of T5αH was, however, found to be very low, because only up to 2.5% of the taxa-4(5),11(12)-diene olefin was converted to 5α-hydroxytaxa-4(20),11(12)-diene in engineered yeast co-expressing both TS and T5αH (8).

Recently tobacco glandular trichome cells were described as an attractive and efficient target for the engineering of diterpe-

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1 The abbreviations used are: GGPP, geranylgeranylpyrophosphate; CBT, cembraetriol-ol; CBTS, cembraetriol-ol synthase; FT, flow through; ihp, intro hairpin RNA interference; MS, mass spectrometry; OCT, 5(12)-oxa-3(11)-cyclotaxane; T5αH, taxa-4(5),11(12)-diene 5α-hydroxylase; TS, taxa-4(5),11(12)-diene synthase; GC, gas chromatography; HPLC, high pressure liquid chromatography; HP-TLC, high performance TLC.
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noid biosynthesis (18, 19). Because of significant knowledge of the paclitaxel biosynthetic pathway accumulated over the years, it appeared a good model to test the ability of the tobacco trichomes to support the production of complex diterpenoids.

Nicotiana sylvestris is a wild tobacco whose trichomes produce a single class of diterpenoids, the cembratrien-diols (CBT-diols) (20). The biosynthetic pathway of these compounds was unraveled recently (21), making it possible to knock out their production by gene silencing. This was achieved by introducing an intron hairpin RNA interference construct, named ihpCBTS, targeting the first step of the CBT-diols pathway, cembratrienol synthase (CBTS). As described elsewhere (11), ihpCBTS under control of the CBTS trichome-specific promoter led to a drastic reduction in the production of endogenous CBT-diols to less than 10% of wild-type levels. The ihpCBTS line offers an excellent platform for expression of heterologous diterpene synthases.

As a preliminary step to this study, TS was successfully expressed in N. sylvestris trichome cells yielding taxa-4(5),11(12)-diene at \( \sim 20 \, \mu g g^{-1} \) of fresh leaves on average, 10% of which is secreted and recovered in the trichome exudates (11).

This study reports the co-expression of both TS and CYP725A4, the next presumed step in the paclitaxel pathway, (11). Unexpectedly, instead of 5\( \alpha \)-CYP725A4, the next presumed step in the paclitaxel pathway, (11), of which is secreted and recovered in the trichome exudates involving oxidation and subsequent cyclizations.

We propose an unprecedented CYP-mediated reaction sequence for the conversion of taxa-4(5),11(12)-diene to this new compound, characterized. We describe here how this new compound was identified, purified, and chemically characterized. We propose an unprecedented CYP-mediated reaction sequence for the conversion of taxa-4(5),11(12)-diene to this new compound, involving oxidation and subsequent cyclizations.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—[1-\(^{3}H\)]GGPP (15 Ci/mmol) and PD-10 column were from General Electric Healthcare. GGPP, NADPH, dithiothreitol, antibiotics, salts and buffers were from Sigma. All of the solvents were purchased from Fisher except diethylether, which was from Sigma. HP-TLC silica gel 60 plates (10 \( \times \) 10 cm) were from Merck, and prepacked silica columns were from Interchim. Films (catalog number MS 111 1681) and reagents for autoradiography were from Kodak. Taxa-4(5),11(12)-diene, 5\( \alpha \)-hydroxytaxa-4(20),11(12)-diene, and 5\( \alpha \)-acetoxytaxa-4(20),11(12)-diene standards were provided by Rodney Croteau.

**Plant Culture**—Tobacco plants were grown in soil in climate-controlled chambers, with a 14-h day/10-h night photoperiod under an illumination of 400-W high pressure metal halide lights, a temperature cycle of 25/20 °C (day/night), and 65% relative humidity. The plants were irrigated once a day with deionized water under an illumination of 400-W high pressure metal halide lights, a temperature cycle of 25/20 °C (day/night), and 65% relative humidity. The plants were irrigated once a day with deionized water.

**Plant Transformation and Progeny**—The three pLibro vectors were separately introduced into the Agrobacterium strain LBA4404 by electroporation and then into the genome of N. sylvestris via standard tobacco transformation methods (24). Transformants and their progeny were selected by real time PCR to determine the copy number and expression level of transgenes. Real time PCR was carried out with TaqMan probes (ABI) in duplex reactions, on an ABI7000 instrument. The TaqMan reference probe matched one allele of the CYP71D16 gene (18), which was shown to be a single copy gene in N. sylvestris and expressed specifically in the trichome cells (data not shown). The relative expression level was determined from the ratio 2\(^{-}\Delta\Delta Ct}\) CYP71D16/2\(^{-}\Delta\Delta Ct}\) transgene. Transgenic plants were crossed with TaqMan probes (ABI) in duplex reactions, on an ABI7000 instrument. The TaqMan reference probe matched one allele of the CYP71D16 gene (18), which was shown to be a single copy gene in N. sylvestris and expressed specifically in the trichome cells (data not shown). The relative expression level was determined from the ratio.

**Production of Truncated TS in Escherichia coli**—The TS cDNA coding for a protein truncated at the N terminus at amino acid position 75 (TS_trunc) was cloned in the pET-30b expression vector (Novagen) between the Ndel and EcoRI restriction sites. The stop codon was removed to insert a histidine tag in frame at the C terminus. The resulting recombinant vector pET::TS_trunc-HisTag was introduced into BL21-CodonPlus(DE3)-RIL cells (Stratagene). The transformed cells were routinely grown at 37 °C to an A\(_{600}\) of 0.6 in LB medium with 100 \( \mu \)g/ml ampicillin, 50 \( \mu \)g/ml chloramphenicol, and 20 \( \mu \)g/ml tetracycline. The cells were harvested by centrifugation and washed in a lysate buffer (200 mM potassium phosphate, pH 7.8, 10 mM imidazole, 5 mM dithiothreitol, 10% (v/v) glycerol, 200 mM KCl). The cells were disrupted in a French Press. The extract was cleared by centrifugation (10,000 \( \times \) g, 15 min), and
the supernatant was desalted on a PD-10 column equilibrated with the lysis buffer. Purification of the recombinant TS_trunc-HisTag was performed using nickel-nitritotriacetic acid resin according to the manufacturer’s protocol (Qiagen). The final elution buffer containing proteins was exchanged with assay buffer (20 mM Hepes, pH 7.8, 20% (v/v) glycerol, 100 mM KCl, 7.5 mM MgCl₂, 1 mM dithioerythritol, using a PD-10 column).

Production of [2-3H]Taxa-4(5),11(12)-diene—[2-3H]Taxa-4(5),11(12)-diene was produced using purified recombinant TS_trunc-HisTag. [1-3H]GGPP (15 μCi/nmol) was diluted 28 times with cold GGPP to a final specific radioactivity of 0.54 μCi/nmol at a concentration of 1 mM. Production of [2-3H]taxa-4(5),11(12)-diene was achieved using 500 μg of TS_trunc-HisTag, 92.7 μM [1-3H]GGPP at 30 °C during 2 h. The reaction medium was extracted with pentane (3 times, v/v), and the product was passed through a 100 mg silica column, concentrated, and counted in a scintillation counter.

Labeling Experiment—The [2-3H]taxa-4(5),11(12)-diene was resuspended in 5 mM potassium phosphate buffer, pH 5.5, 10 mM sucrose, 2 mM KCl, 0.2 mM EDTA, 0.05% (v/v) Tween 20 and sonicated 5 min in a bath. 15 discs (1-cm diameter) of fresh half-expanded leaves were vacuum infiltrated with the tritiated buffer containing 1 μCi of [2-3H]taxa-4(5),11(12)-diene in a 5-ml glass tube. The tissues were incubated 18 h at 25 °C under light and at 80 rpm (Infors incubator). After incubation the discs were washed with cold phosphate buffer and frozen in liquid nitrogen. The frozen discs were ground with mortar and pestle to a fine powder and thawed in a ternary blend of chloroform:methanol:water (0.5:1.0:4.0 v/v/v) according to Ref. 25. The organic phase was separated by adding chloroform (0.5v) and water (0.5v) and dried under a stream of nitrogen. The extract was dissolved in pentane and loaded onto a 200-mg silica column. The column was eluted with diethylther. The flow through and eluate were brought to dryness under a stream of nitrogen and redissolved in 20 μl of pentane, and 10 μl was deposited onto a HP-TLC silica gel 60 plate. The plate was developed with a pentane:ethyl acetate film of HP-1ms (Agilent Technologies). Separation was done in a 30-m × 0.25-mm diameter capillary with 0.25-μm film of HP-1ms (Agilent Technologies). The samples were injected using a cool on-column injector at 40 °C, followed by 10 °C/min to 150 °C, 2 min hold. The oven was programmed to start at 40 °C and then increased at 10 °C/min to 100 °C, followed by 3 °C/min to 280 °C and a 5-min hold. Electronic impact was recorded at 70 eV. OCT was quantified by ionization yield of diterpene alcohol: cis-abienol (Librophyt internal reference).

GC-MS Analysis—Trichome exudate was extracted from fresh tobacco leaves in pentane for 2 min, and the samples were injected directly into GC-MS for analysis on a 6890 N gas chromatograph coupled to a 5973 N mass spectrometer (Agilent Technologies). Separation was done in a 30-m × 0.25-mm diameter capillary with 0.25-μm film of HP-1ms (Agilent Technologies). The samples were injected using a cool on-column injector at 40 °C, followed by 10 °C/min to 150 °C, 2 min hold. The oven was programmed to start at 40 °C and then increased at 10 °C/min to 100 °C, followed by 3 °C/min to 280 °C and a 5-min hold. Electronic impact was recorded at 70 eV. OCT was quantified by ionization yield of diterpene alcohol: cis-abienol (Librophyt internal reference).

GC-MS Analysis—Production of the NsTax Tobacco Transgenic Line Expressing TS and CYP725A4—To produce the 5α-hydroxytaxa-4(5),11(12)-diene in the trichomes of N. sylvestris, the cDNAs encoding TS (15) and CYP725A4 (16) were introduced in independent plant expression cassettes. The TS cDNA is controlled by the CBTS promoter (23), and the CYP725A4 cDNA is controlled by the CYP71D16 promoter, both of which are trichome-specific (18). Both constructs were stably introduced in the genome of N. sylvestris ihpCBTS line. In previous work, we have shown that expression of TS leads to efficient production of taxa-4(5),11(12)-diene in the leaf exudates (11). A succession of crosses and self-fertilization gave a transgenic N. syl-
The vestris line named NsTax harboring and expressing the two transgenes in the homozygous state. Genotypes of transgenic tobacco lines and expression in NsTax plants were determined by qPCR. DNA and RNA extractions and qPCR were done in triplicate. Expression levels are calculated using the CYP71D16 (trichome-specific gene) as reference. copy nb, 1 = one transgene per genome, 2 = homozygous state; exp, RNA expression level.

| Plants   | TS copy nb | TS exp | CYP725A4 copy nb | CYP725A4 exp | ihpCBTS copy nb | NsTax exp |
|----------|------------|--------|------------------|--------------|-----------------|-----------|
| TS       | 1          | 6 ± 1  | 1                | 68 ± 15      | 1               | 6 ± 2     |
| CYP725A4 | 1          | 68 ± 15| 1                | 68 ± 15      | 1               | 80 ± 19   |
| ihpCBTS  |            |        |                  |              |                 | 1         |
| NsTax    | 2          | 6 ± 2  | 2                | 80 ± 19      | 2               | 19        |

Production of a Novel Oxidized Taxane in NsTax Plants—
Leaf exudate from transgenic plants was analyzed by direct injection with a cool on-column injector into a gas chromatograph coupled to a mass spectrometer (GC-MS), thereby avoiding any molecule alteration that could have occurred upon sample preparation (i.e. silica beads) or high temperature injection. Taxa-4(5),11(12)-diene and 5α-hydroxytaxa-4(20),11(12)-diene standards were used as references (Fig. 1A). Leaf exudates of ihpCBTS plants had no detectable taxanes (Fig. 1B). Consistent with previous work (11), plants expressing TS and ihpCBTS produced taxa-4(5),11(12)-diene in the leaf exudate at 2 g/g fresh weight (Fig. 1C). When CYP725A4 was expressed alone, no new product could be detected in the exudate (Fig. 1D). Finally, when TS, CYP725A4, and ihpCBTS were co-expressed in NsTax plants, taxa-4(5),11(12)-diene was absent from the GC-MS profile, but the 5α-hydroxytaxa-4(20),11(12)-diene could not be detected either. Instead a new isomer, which was named OCT and whose structure is described below, was detected (Fig. 1E). Because of the lack of standards for taxane diterpenoids with a low level of oxidation, the OCT could not be identified initially. The retention time of OCT (39.52 min) was between those of taxa-4(5),11(12)-diene (36.86 min) and 5α-hydroxytaxa-4(20),11(12)-diene (40.72 min), suggesting an intermediate volatility. The mass spectrum of the OCT was highly distinct with a major positive ion m/z 191 (Fig. 1F) and a putative molecular ion m/z 288. This latter value is the expected molecular weight of a mono-oxidized diterpene, consistent with a single oxidative reaction on the taxadiene core. At this stage, exudate analysis and molecular weight were the only evidence pointing to OCT as an oxidized taxoid.

**OCT as a New Taxoid**—To unambiguously prove that OCT is derived from taxa-4(5),11(12)-diene, tritiated taxa-4(5),11(12)-diene standards were used as references (Fig. 1G). Leaf exudates of NsTax plants were infiltrated with [2-3H]taxa-4(5),11(12)-diene. After incubation, organic soluble compounds were extracted and passed through a silica column. The FT and the eluate (E) were deposited on a silica plate, which was developed by autoradiography. Each labeling experiment was repeated twice. WT, wild type plant; TS, plant harboring the taxa-4(5),11(12)-diene synthase; NsTax, plant harboring TS, CYP725A4, and ihpCBTS. A, taxa-4(5),11(12)-diene; B, 5α-acetoxytaxa-4(20),11(12)-diene; C, 5α-hydroxytaxa-4(20),11(12)-diene; D, deposit. Table 1. Genotypes of transgenic tobacco lines and expression in NsTax plants

| Plants   | TS copy nb | TS exp | CYP725A4 copy nb | CYP725A4 exp | ihpCBTS copy nb | NsTax exp |
|----------|------------|--------|------------------|--------------|-----------------|-----------|
| TS       | 1          | 6 ± 1  | 1                | 68 ± 15      | 1               | 6 ± 2     |
| CYP725A4 | 1          | 68 ± 15| 1                | 68 ± 15      | 1               | 80 ± 19   |
| ihpCBTS  |            |        |                  |              |                 | 1         |
| NsTax    | 2          | 6 ± 2  | 2                | 80 ± 19      | 2               | 19        |
4(5),11(12)-diene was followed in trichome cells. [2-\textsuperscript{3}H]Taxa-4(5),11(12)-diene was biochemically synthesized using truncated TS recombinant protein from \textit{E. coli} and [1-\textsuperscript{3}H]GGPP as substrate. In our experimental conditions, labeling revealed that 10% of [1-\textsuperscript{3}H]GGPP was converted to [2-\textsuperscript{3}H]taxa-4(5),11(12)-diene. The [2-\textsuperscript{3}H]taxa-4(5),11(12)-diene was then used to vacuum infiltrate discs of fresh tobacco leaves. After overnight incubation, organic soluble compounds were extracted from whole leaves and loaded onto a silica resin to separate olefins, which were retrieved in the flow through (FT), from eluted oxidized compounds. FT and eluates were then analyzed on HP-TLC plates developed by autoradiography.

When infiltration was done on leaves from wild type or TS-expressing plants, no radioactive label could be detected except in the FT (Fig. 2), indicating that [2-\textsuperscript{3}H]taxa-4(5),11(12)-diene was not metabolized. In contrast, with NsTax leaves radioactive label was recovered in the FT (60%) but also in the eluate (40%) (Fig. 2), meaning that [2-\textsuperscript{3}H]taxa-4(5),11(12)-diene was converted to a tritiated oxidized taxane. The labeled eluate revealed a band on the TLC radioautograph near the migration front with a retention factor of 0.95 (Fig. 2). This band was eluted from the silica layer and analyzed by GC-MS. The chromatogram showed a peak with a retention time and mass spectrum precisely identical to those of OCT (Fig. 1, E and F), demonstrating that the previously identified OCT from the exudate is indeed an oxidized taxoid. HP-TLC analysis also showed that OCT (retention factor of 0.95) did not co-migrate with either of the known taxoid standards, 5α-hydroxytaxa-4(20),11(12)-diene (retention factor of 0.28) or 5α-acetoxytaxa-4(20),11(12)-diene (retention factor of 0.88). Therefore, OCT is a very hydrophobic taxoid possibly bearing an epoxide or an ether function, in agreement with a molecular weight of 288.

**Purification of OCT**—Purification of OCT was carried out to determine its structure. Fresh leaf exudates from NsTax plants contained 2 μg g\textsuperscript{-1} of OCT, which represented 0.4% (w/w) of the exudate. This low percentage was mainly due to the presence of high quantities of waxes and tocopherol. Chromatography showed a peak with a retention time and mass spectrum precisely identical to those of OCT (Fig. 1, E and F), demonstrating that the previously identified OCT from the exudate is indeed an oxidized taxoid. HP-TLC analysis also showed that OCT (retention factor of 0.95) did not co-migrate with either of the known taxoid standards, 5α-hydroxytaxa-4(20),11(12)-diene (retention factor of 0.28) or 5α-acetoxytaxa-4(20),11(12)-diene (retention factor of 0.88). Therefore, OCT is a very hydrophobic taxoid possibly bearing an epoxide or an ether function, in agreement with a molecular weight of 288.

**TABLE 2**

| Steps          | Enrichment | Step yield | Total yield | OCT quantities |
|----------------|------------|------------|-------------|----------------|
| Exudate        | 0.4        | %          | %           | %              |
| Silica column  | 4          | 66         | 66          | 1.05           |
| HPLC           | 95         | 56         | 37          | 0.56           |

**TABLE 3**

| Complete \textsuperscript{1}H and \textsuperscript{13}C NMR assignments for OCT and correlation table (CDCl\textsubscript{3}) |
|---|---|---|---|---|
| **Position** | \(\delta_{\text{HM}}\)\textsuperscript{a} | COSY/TOCSY | HMQC | HMBC | \(\delta_{\text{HM}}\)\textsuperscript{b} |
| 1   | 1.71, dd, (8.4, 5.0) | 2h, 14b | 45.9 | 11 | 45.9 |
| 2a  | 1.33, d, (12.9) | 2b | 39.1 | 1, 3, 4, 8, 11, 14, 15 | 39.1 |
| 2b  | 2.21, dd, (12.9, 5.0) | 1, 2a | 39.1 | 1, 3, 4, 8, 14, 15 | 39.1 |
| 3   | 2.47, qdl, (7.0, 3.4) | 5, 6a, 20 | 37.1 | 5 | 37.1 |
| 4   | 3.97, dd, (9.1, 3.4) | 4, 6a, 6b, 7a, 20 | 69.8 | 37.5 | 69.8 |
| 6a  | 1.83, m | 30.2 | 30.2 |
| 6b  | 2.04, m | 30.2 | 30.2 |
| 7a  | 1.36, d, (10.9) | 37.5 | 6, 9 | 37.5 |
| 7b  | 1.83, m | 37.5 | 37.5 |
| 8   | 1.53, dd, (12.7, 8.8) | 47.3 | 3, 7, 11 | 47.3 |
| 9a  | 1.82, m | 47.3 |
| 9b  | 1.31, dd, (9.6, 3.6) | 30.2 | 3, 8, 11, 15 | 30.2 |
| 10a | 1.38, ddd, (10.9, 3.6, 1) | 30.2 | 8, 11, 15 | 30.2 |
| 11  | 1.98, m | 66.0 |
| 12  | 1.84, m | 80.5 |
| 13b | 1.98, m | 36.4 |
| 14a | 1.62, ddd, (14.3, 11.2, 5.2) | 1 | 28.1 | 1 | 28.1 |
| 14b | 2.01, m | 28.1 | 1, 11, 12, 15 | 28.1 |
| 15  | 0.93, brs | 28.6 | 1, 11, 15, 17 | 28.6 |
| 16  | 1.01, brs | 26.9 | 1, 11, 15, 16 | 26.9 |
| 17  | 1.19, s | 30.3 |
| 18  | 1.04, s | 28.0 | 3, 7, 8, 9 | 28.0 |
| 19  | 1.13, d, (7.0) | 15.2 | 4, 5, 6a, 6b |
| 20  | | 3, 4, 5 | 15.2 |

\textsuperscript{a} The values were recorded at 500 MHz. s, singlet; brs, broad singlet; d, doublet; q, quadruplet; m, multiplet. (J in Hz).

\textsuperscript{b} The values were recorded at 125 MHz.
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phy on a silica column brought a 10-fold enrichment of OCT. The main contaminant still present at that stage was tocopherol. A nearly total purification of OCT was achieved by HPLC. The final fraction of OCT showed a chromatographic purity of 95%. The purification protocol is described under “Experimental Procedures.” Purification data are compiled in Table 2. In fine, 600 μg of OCT were recovered from the exudate of 800 g of fresh leaves. This was sufficient for structural analysis.

NMR Structure of OCT—Purified OCT (Fig. 3B) was subjected to NMR analysis. To simplify interpretation of the spectra, only differences between OCT and taxa-4(5),11(12)-diene (Fig. 3A) are presented here. 1H and 13C NMR chemical shifts and two-dimensional correlation data are summarized in Table 3. As expected for taxane cores, protons from the methyl groups CH3-16, CH3-17, CH3-18, and CH3-19, all characterized by a singlet, were readily assigned from the 1H NMR spectrum (Table 3). In contrast to NMR data from the reference taxa-4(5),11(12)-diene structure (Fig. 3A), the CH3-20 methyl protons appeared as a doublet (δ 1.13, J 7) (Table 3) and showed a cross-peak in the COSY spectrum at δ 2.47 corresponding to a CH-4 methine proton (Fig. 4A), which resonated as a doublet (J 7.0 and 3.4) in the 1H NMR spectrum (Table 3). Moreover the CH-4 methine proton showed a cross-peak in COSY spectrum at δ 3.97 corresponding to a methine proton.

FIGURE 4. Structural analysis of OCT. A, 1H/1H two-dimensional NMR COSY spectrum. The arrows indicate the correlations between the CHα-2 proton (δ 1.33 ppm) and the CHα-10 proton (δ 1.31 ppm) with the C-3 (δ 53.3 ppm).

B
CH-5 (Fig. 4A), which resonated as a double doublet (J 9.1 and J 3.4) in the 1H NMR spectrum (Fig. 4A and Table 3). Deshielded resonances of CH-5 at δ 3.97 in 1H NMR spectrum and δ 69.8 in 13C NMR spectrum, as determined by a cross-peak in the HMBC spectrum, were characteristic of an oxygen-bearing carbon atom (Table 3). Only C-5 can be bound to both one proton and one oxygen. Indeed, C-3, which is already involved in three C-C bonds to the taxane core, cannot accept those two additional bonds. Two conclusions can be drawn for this side of the taxane core: (i) C-5 is oxidized and protonated and (ii) C-4 is protonated (Fig. 3B). Thus, the 4(5) double bond of taxa-4(5),11(12)-diene core is modified to a single bond linkage. Both the lack of correlation in the HMBC spectrum for C-3 (δ 53.3), C-11 (δ 66.0), and C-12 (δ 80.5) and their chemical shifts are indicative of sp3 quaternary carbons (Table 3). This means that, compared with taxa-4(5),11(12)-diene, the sp2 double bond 11(12) has been modified to one single bond linkage (Fig. 3B). As for C-5, C-12 has a deshielded resonance because of an oxidized carbon (δ 80.5) (Table 3). Because the molecular weight of 288 (Fig. 1F) for OCT indicates a molecule containing only one oxygen atom, C-5 and C-12 must be linked via an ether function (Fig. 3B). Because C-3 and C-11 are quaternary carbons, it can be concluded that for a molecular weight of 288 a C-3/C-11 linkage (Fig. 3B) has to be considered. This assumption is validated by the HMBC spectrum in which one methylene proton CH2-2 (δ 1.33) is correlated with C-11 (δ 66.0) and one methane proton CH2-10 (δ 1.31) is correlated with C-3 (δ 53.3) (Fig. 4B). In conclusion, the OCT is a 5(12)-oxa-3(11)-cyclotaxane with five fused rings (Fig. 3, B–D). The three-dimensional model of OCT displays a globular shape in which the oxygen atom of the ether function is partially hidden inside the structure (Fig. 3D). This last feature may explain why OCT is very stable (up to 200 °C), insensitive to air oxidation, and non-volatile in standard conditions.

Expression of CYP725A4 in Yeast—According to Ref. 16, recombinant CYP725A4[ext.1] from yeast or S9 insect cells is reported to transform taxa-4(5),11(12)-diene to 5α-hydroxytaxa-4(20),11(12)-diene, whereas recombinant tobacco CYP725A4 with the same substrate produced OCT. To check whether the slight difference of sequence in the C terminus between the two enzymes (see “Experimental Procedures”) is responsible for the difference in catalytic activities, both of them were expressed in yeast. Yeast microsomes expressing CYP725A4 converted taxa-4(5),11(12)-diene to OCT with a substrate conversion rate of 96 ± 1% in our experimental conditions (Fig. 5B; enzymatic assays were repeated five times). The affinity constant Km of taxa-4(5),11(12)-diene for CYP725A4 was 6.9 ± 0.4 μM. Under the same conditions, yeast microsomes expressing CYP725A4[ext.1] also catalyzed the same conversion of taxa-4(20),11(12)-diene to OCT with an identical efficiency (5 replicates) (Fig. 5C). Importantly, 5α-hydroxytaxa-4(20),11(12)-diene was never detected in the reaction product, even in the highly sensitive single ion monitoring mode. The chromatogram showed a very minor peak at 41.47 min, but its mass spectrum and retention time did not match those of 5α-hydroxytaxa-4(20),11(12)-diene (40.72 min).

In conclusion, these results demonstrated that (i) the sequence difference between CYP725A4[ext.1] (T5αH) and CYP725A4 has no effect on the nature of the reaction product, and more importantly (ii) recombinant CYP725A4 whether expressed in yeast or tobacco showed the same catalytic activity and was able to trigger the complete rearrangement cascade of taxa-4(5),11(12)-diene into the complex OCT molecule, without implication of any other enzyme in this process.

**DISCUSSION**

The present work demonstrates that CYP725A4 is a P-450 oxygenase able to oxidize the taxa-4(5),11(12)-diene into the OCT. This oxidation involves the addition of one oxygen to the molecule concomitant with an important reorganization of the structure featuring an additional C-3/C-11 bond dividing the eight-carbon cycle of the taxa-4(5),11(12)-diene scaffold in two fused five-carbon rings and an unusual C-5/C12 ether bridge spanning the entire molecule (Fig. 3). This confirms previous structural information of the taxane core where the C-5 and C-12, despite being at opposite ends of the molecule, are neighbors because of the U-shape of the molecule (30).

The combined oxidation and cyclization activity of CYP725A4 in tobacco (Figs. 1 and 2) was confirmed by heterologous expression in yeast (Fig. 5). This shows that the formation of OCT is due to the sole catalytic activity of CYP725A4. No trace of 5α-hydroxytaxa-4(20),11(12)-diene could be found in either the tobacco or the yeast experiments. Thus, we were unable to reproduce the findings of the first report on the activity of CYP725A4 (16) where a taxa-4(5),11(12)-diene 5α-hydroxylase activity of the yeast and S9 recombinant enzymes was described. This difference will be discussed below in relation to the putative CYP725A4 reaction mechanism.

The extensive modifications leading to the conversion of taxa-4(5),11(12)-diene to OCT suggests a complex and unusual
reaction mechanism. Drawing from the literature on the CYP oxidation cycle (31, 32), the following scheme is proposed (Fig. 6): (i) The reactive high valent FeV = O forms a σ complex by oxidation of the 4(5) π systems of taxa-4(5),11(12)-diene. The newly created C=4 radical gives rise to the C=4 carbocation by electronic displacement to the heme decreasing its oxidation level from FeIV to FeIII. (ii) The next step involves the hydride migration from C-3 to C-4, creating the C-3 carbocation. (iii) Electronic displacement from the double bond 11(12) to the electrophilic C=3 creates the C=3/C=11 bond, thus forming a new carbocation C=12. (iv) Finally, the σ intermediate collapses to release the OCT harboring the 5(12) cyclic ether. In this scheme, the C=4 carbocationic form is favored over the C-4 radical because the migration of a radical across the entire taxane core (nonaromatic system) is unlikely (31). Additionally, hydride shift of step (ii) is also more coherent with the carbocationic model (31).

A similar reaction sequence involving a carbon radical, an electronic rearrangement to a carbocation form, and a final cyclization, has been described for the metabolism of capsaicin by a P-450 enzyme (33). Carbon/carbon intermolecular bonding for flavilin biosynthesis and intramolecular cyclic ether formation for vancomycin biosynthesis, all triggered by CYP enzymes, have been recently reviewed (32). Hydride migration has also been reported along with oxidation of olefins (31). Therefore, although each individual step described above for OCT biosynthesis is not novel by itself, the combination of all these steps in an ordered fashion by the single CYP725A4 enzyme is without precedent in the literature.

4(5)-Epoxy-taxa-11(12)-ene may be considered as an alternative intermediate. However, despite the recent implication of an epoxide in the somewhat related formation of pyranoid- and furanoid-linalool by CYP2D6 (34), this hypothesis is unlikely because the subsequent cyclization steps would require opening of the epoxide by a strong Lewis acid, which metalloporphyrins cannot do (31).

As mentioned above, our findings disagree with the first report describing the activity of CYP725A4. The expression in yeast of both the original and the corrected sequences ruled out the slight amino acid difference at the C terminus of CYP725A4 as a possible explanation (Fig. 5). In the previously published work, the His6 tag added at the C terminus may also explain a

**FIGURE 6. Proposed reaction mechanism for the catalytic conversion of taxa-4(5),11(12)-diene by CYP725A4.** The reactive FeV = O of CYP725A4 acts first to form a σ (FeIV) complex and a C-4 radical. The electronic displacement forms the C=4 carbocation and the heme reduction into FeIII. Hydride migration and electronic displacements lead to the sequential formation of C=3 and C=12 carbocations, before final collapse of the σ complex to form the cyclic ether of OCT.
modified activity. Another explanation may be found in the use of radiolabeled substrates, namely [20-2H3]taxa-4(5),11(12)-diene (17) or [20-3H3]taxa-4(5),11(12)-diene (8, 16, 17), in the initial characterization of CYP725A4 by Croteau and co-workers (16). Isotopic effects on P-450 enzymes are known to cause intramolecular competition leading to different products (35). Accordingly, the radioisotopes used may have prevented the normal sequence of electronic movements, leading to a diversion of the reaction. Most probably, at the C+4 carboxylation intermediate proposed in our model, the presence of isotope on the C-20 methyl may drive an elimination of a C-20 proton, forming the allylic radical required to explain subsequent C-5α-hydroxylation, as reported (16).

Although OCT has not been detected in yew species, related taxoids with a C-3/C-11 bridge, also known as cyclotaxanes, have been described (36–41). None of these 3(11)-cyclotaxanes has a 5(12) cyclic ether. However, another cyclotaxane with a 4(13) cyclic ether bond was recently identified from *Taxus canadensis* needles (41). These observations tend to confirm that the cyclase activity of CYP725A4 or closely related enzymes is biologically relevant in the biosynthesis of cyclotaxane derivatives in yew trees. Nonetheless, it is clear that OCT is probably not an intermediate of the paclitaxel pathway because the disruption of the 3(11) bond to regenerate the taxa-11(12)-ene core seems highly improbable. Rather, OCT is likely to be the entry point of a metabolic branch of the highly diverse taxoid biosynthesis. Whether taxa-4 (20),11(12)-diene is the actual substrate of CYP725A4 in yew cells remains to be determined, and it is possible that different taxane substrates could yield different cyclization patterns as observed for other cyclotaxanes.

Overall, this report also illustrates the potential of tobacco trichomes as a relevant target for the engineering of diterpenes. The complete conversion of taxa-4(5),11(12)-diene to OCT in *planta* demonstrates that the P-450 reductase from tobacco trichomes interacts perfectly well with the CYP725A4 from yew and that NADPH is an available and nonlimiting co-substrate in these cells. Thus, tobacco trichomes are a remarkable platform for pathway discovery and compound production in diterpenoid metabolism.

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**Biosynthesis of 5(12)-Oxa-3(11)-cyclotaxane**

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