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A Cytochrome P-450 Monooxygenase Catalyzes the First Step in the Conversion of Tabersonine to Vindoline in *Catharanthus roseus*[1]

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Hydroxylation at the C-16 position of the indole alkaloid tabersonine has been suggested as the first step toward vindoline biosynthesis in *Catharanthus roseus*. Tabersonine 16-hydroxylase (16-OH) activity was detected in total protein extracts from young leaves of *C. roseus* using a novel coupled assay system. Enzyme activity was dependent on NADPH and molecular oxygen and was inhibited by CO, clotrimazole, miconazole, and cytochrome *c*. 16-OH was localized to the endoplasmic reticulum by linear sucrose density gradient centrifugation. These data suggest that 16-OH is a cytochrome P-450-dependent monooxygenase. The activity of 16-OH reached a maximum in seedlings 9 d postimbibition and was induced by light. The leaf-specific distribution of 16-OH in the mature plant is consistent with the localization of other enzymes in the tabersonine to vindoline pathway. However, in contrast to enzymes that catalyze the last four steps of vindoline biosynthesis, enzymes responsible for the first two steps from tabersonine (16-OH and 16-*O*-methyltransfersase) were detected in *C. roseus* cell-suspension cultures. These data complement the complex model of vindoline biosynthesis that has evolved with respect to enzyme compartmentalization, metabolic transport, and control mechanisms.

Catharanthus roseus is the source of two commercially important molecules used in cancer treatment, vinblastine and vincristine. These are dimeric indole alkaloids that are formed in vivo by condensation of vindoline and catharanthine. The low yield of dimeric indole alkaloids from the plant (approximately 0.0005%) and their consequent high price have stimulated numerous efforts to develop alternative strategies for their production (Kurz et al., 1985; Petiard et al., 1985; De Luca and Kurz, 1988). Despite these intense efforts, attempts to produce antitumor alkaloids from *C. roseus* cell cultures have failed (reviewed by Van der Heijden et al., 1989) and have been attributed to their inability to synthesize the precursor vindoline. However, the ability of certain cell lines to accumulate high levels of catharanthine and serpentine indicates that they have a high potential for the production of some monoterpenoid indole alkaloids (Kutney et al., 1980a; Stöckigt and Soll, 1980; Deus-Neumann and Zenk, 1984).

These results have prompted a detailed investigation of the enzymology and the regulation of vindoline biosynthesis. Studies from our group (De Luca et al., 1986) have established that tabersonine is transformed to vindoline through a sequence of six enzymatic steps (Fig. 1). These constitute the late stages of vindoline biosynthesis, which appear to be absent from *C. roseus* cell cultures (De Luca et al., 1987). Several enzymes and intermediates in this pathway have been characterized. These enzymes include an *O*-methyltransferase (Fahn et al., 1985b), an *N*-methyltransferase (De Luca et al., 1987), a 2-oxoglutarate-dependent dioxygenase (De Carolis et al., 1990), and an *O*-acetyltransferase (De Luca et al., 1985; Fahn et al., 1985a). Most of the intermediates required for these enzymatic steps have been identified in etiolated seedlings (Balsevich et al., 1986; De Luca et al., 1986). The late stages of the vindoline pathway appear to be highly regulated and are expressed in a development-specific, tissue-specific, and light-dependent manner in germinating seedlings (De Luca et al., 1986, 1988; De Carolis et al., 1990; Aerts and De Luca, 1992). This strict regulation of the pathway might explain its lack of expression and the lack of vindoline accumulation in cell cultures.

The enzymatic conversion of tabersonine appears to involve a precise sequence of reactions that includes aromatic hydroxylation at position 16, 16-*O*-methylation, hydration of the 2,3 double bond, *O*-methylation, hydroxylation at C-4, and 4-*O*-acetylation (De Luca et al., 1986). In our continuing work on the enzymology of vindoline biosynthesis, we report here the characterization of the enzyme responsible for the conversion of tabersonine to 16-hydroxytabersonine and show it to be a microsomal Cyt P-450-dependent monooxygenase. In agreement with the developmental regulation proposed for this part of the vindoline pathway, the enzyme is found in young leaves of the intact plant and is developmentally regulated and light regulated in germinating seedlings.

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Abbreviations: AdoMet, *S*-adenosyl-L-Met; CCR, antimycin A-resistant NADH Cyt *c* reductase; DAT, acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase; EtOAc, ethylacetate; IC_{50}, 50% inhibitory concentration; MeOH, methanol; NMT, *S*-adenosyl-L-Met: 2,3-dihydro-3-hydroxytabersonine-*N*-methyltransferase; 4-OH, desacetoxyvindoline 4-hydroxylase; 16-OH, tabersonine 16-hydroxylase; S_{100}, soluble protein fraction.

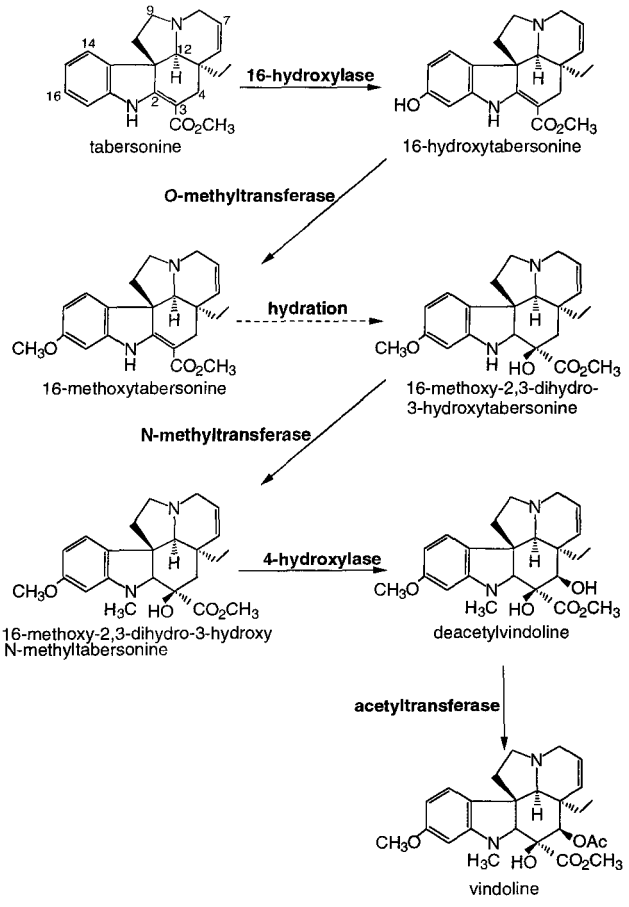

Figure 1. Proposed biosynthesis of vindoline from tabersonine in *C. roseus*. The numbering system used was as for aspidospermidine alkaloids in Chemical Abstracts (1987–1991).

MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-[*methyl*-^{14}C]Met (2.07 GBq/mmol) was from Amersham. Nonidet P-40 (membrane research grade), Cyt *c*, NADH, and NADPH were from Boehringer Mannheim. Oxaloacetate, 1-phenylimidazole, clotrimazole, metyrapone, flusilazole, miconazole, XAD-4 resin, and antimycin A were from Sigma. Piperonylbutoxide was from Aldrich. Cellulysin and macerase were from Calbiochem. Alkaloid substrates were from our reference collection. Tetcyclasis and tricliphane were a kind gift from Dr François Tardif. All other chemicals were of analytical grade.

Plant Materials

Catharanthus roseus (L.) G. Don cv Little Delicata plants were grown under greenhouse conditions. Seedlings were grown as described by Aerts et al. (1994). The *C. roseus* cell-suspension culture (cell line 615) was propagated as described by Eilert et al. (1985).

Preparation of Crude Protein Extract, Microsomal Fraction, and Soluble Protein Fraction

Immature leaves (1–3 cm) from flowering shoots were homogenized with acid-washed sand in 3 mL/g fresh weight 0.1 M Tris-HCl buffer, pH 8.0, and 14 mM β-mercaptoethanol using a mortar and pestle. The slurry was filtered through three layers of cheesecloth, and the filtrate was centrifuged at 700*g* for 4 min at 4°C. The supernatant was desalted on a Sephadex G-25 PD-10 column (Pharmacia), and the crude protein extract was used directly for enzyme assays. Alternatively, the original extract was fractionated into microsomal and soluble proteins by centrifugation at 10,000*g* for 10 min at 4°C to remove large particulate matter. The supernatant was centrifuged at 100,000*g* for 60 min at 4°C, resulting in a green pellet (P100) that contained the microsomal fraction contaminated with thylakoids; this was resuspended in extraction buffer. The supernatant (S_{100}), which represented the total soluble protein, was then desalted on a PD-10 column.

Protein Determination

Protein was determined by the Bradford assay using BSA as standard (Bradford, 1976).

Standard Enzyme Assay

Enzyme assays for 16-OH were performed in a final volume of 100 μL and contained 30 μM tabersonine, 1 mM NADPH, 18 μM (220,000 dpm) *S*-adenosyl-L-[*methyl*-^{14}C]Met, 100 mM Tris-HCl, pH 8.0, 4 mM DTT, and 0.2 to 0.8 mg of protein. The reactions were initiated by the addition of NADPH, whereas assays without NADPH served as controls. After the samples were incubated for 20 min at 30°C, the reactions were terminated by the addition of 100 μL of 2 N NaOH.

The reaction products were extracted twice by vortexing for 1 min with 2 × 500 μL of EtOAc, and each organic phase was collected after it was separated from the aqueous phase by centrifugation at 10,000*g* for 5 min. The EtOAc fractions were pooled, dried in vacuo, redissolved in MeOH containing 5 μg of tabersonine, and subjected to TLC (DC-Plastikfolien Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) using EtOAC-MeOH (90:10, v/v) as the solvent system. Tabersonine and 16-[^{14}C]methoxytabersonine comigrated (R_F 0.65), which facilitated the localization of this radiolabeled product. The chromatograms were either autoradiographed (Kodak XAR-5 film) or visualized with UV light (360 nm). The radioactive and blue fluorescent region of the TLC plates, corresponding to 16-methoxytabersonine, were cut out, placed in scintillation cocktail, and quantified by liquid scintillation spectrometry. 16-OH activity is expressed as the amount of 16-[^{14}C]methoxytabersonine synthesized in the presence of NADPH less that produced in the absence of NADPH. This control assay was used rather than those using boiled enzyme or those without enzyme since its background activities were higher.

The activity obtained in the absence of NADPH reflects the conversion of endogenous substrates, possibly including 16-hydroxytabersonine, which were difficult to remove completely during the different fractionation procedures.

Product Analysis

The major product extracted from the standard enzyme assays was identified from its R_F value by TLC using two different solvent systems (A: EtOAc-MeOH, 90:10; B: hexane-diethylether, 50:50, v/v). Alkaloids were visualized by spraying with ceric ammonium sulfate reagent (Farnsworth et al., 1964). Alternatively, tabersonine and 16-methoxytabersonine were detected by their blue fluorescence under irradiation at 360 nm (Petiard et al., 1980). For UV spectrum analysis, the radioactive product was first separated from tabersonine by TLC (solvent B) and then injected on a reverse-phase C_{18} column (Nova-Pak C_{18}, 3.9 × 300 mm, Millipore; solvent: 75% MeOH, 25% water, 0.1% triethylamine; flow rate: 0.6 mL/min) connected to a Waters 991 photodiode array detector. Fractions of eluted materials were also collected and analyzed by liquid scintillation spectrometry. Under these chromatographic conditions, radioactive product was eluted at 30.5 min, and the UV spectra obtained for this product was identical with that reported previously for authentic 16-methoxytabersonine (Pyuskyulev et al., 1967). In addition, 16-methoxytabersonine extracted and purified from etiolated seedlings also eluted under these conditions at 30.5 min with the same UV spectra.

Inhibitor Assay

To assess the effect of inhibitors of P-450-dependent enzymes, assays were conducted as described above with the omission of DTT from the reaction mixture, and inhibitors were incubated with protein extract for 5 min prior to initiation of the reaction. For experiments with CO, the reaction mixtures were equilibrated with gas mixtures for 10 min in Teflon-sealed Reacti-vials (Pierce) on ice before addition of NADPH.

Isopyknic Suc Density Centrifugation

Protoplasts were prepared from young leaves as described by De Luca and Cutler (1987) and lysed in 330 mM sorbitol, 50 mM Hepes-NaOH, pH 7.0, 0.1 mM PMSF, 2 mM EDTA, and 0.005% Nonidet P-40 using a Potter-Elvehjeim (Thomas Scientific, Philadelphia, PA) grinder. The homogenate was centrifuged at 200*g* for 6 min to remove starch and intact protoplasts. The supernatant (5 mL, 250 μg Chl) was applied to a linear 26 to 65% (w/w) Suc gradient containing 0.05 M Tricine-NaOH buffer, pH 7.5. The gradient was centrifuged for 3 h at 100,000*g* at 5°C in a SW-28 rotor (Beckman). After centrifugation, the bottom of the tube was punctured with a needle (16 gauge) and 1-mL fractions were collected. Marker enzymes and Chl were assayed as described previously (De Luca and Cutler, 1987). 16-OH was assayed as described above except that 50 μL of the S_{100} soluble protein fraction was added to the reaction mixture as a source of *O*-methyltransferase.

RESULTS

Enzymic Hydroxylation of Tabersonine

The first proposed step in the conversion of tabersonine to vindoline involves the hydroxylation of the aromatic ring on C-16 (Balsevich et al., 1986) (Fig. 1). An enzyme assay to measure the production of 16-hydroxytabersonine was developed by coupling it to the second step in the vindoline pathway, which involves an AdoMet-dependent *O*-methylation of this hydroxyl group (Fahn et al., 1985b) (Fig. 2).

The performance of hydroxylase assays with total desalted protein extracts from young leaves of *C. roseus* in the presence of [$^{14}CH_3$]AdoMet resulted in the synthesis of

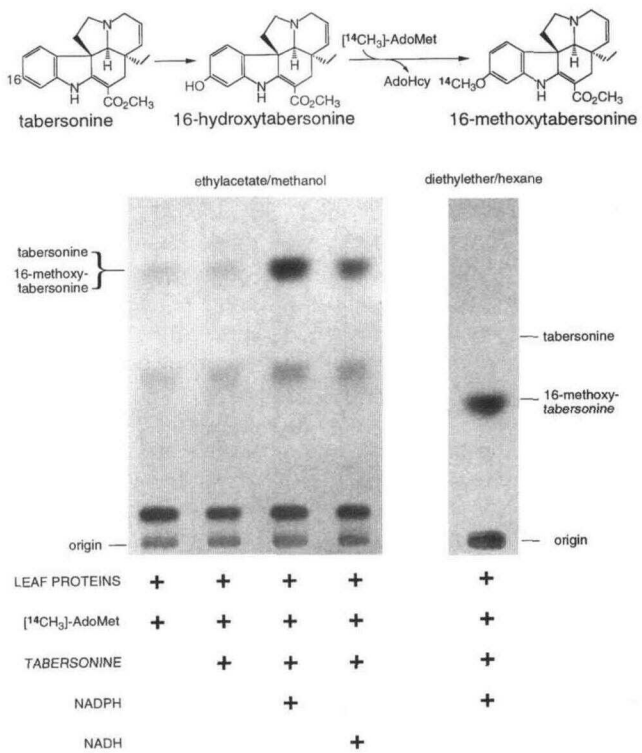

Figure 2. Cofactor and substrate requirements for 16-hydroxylase. 16-Hydroxylase activity was assayed using a coupled enzyme assay. The product of the hydroxylase reaction was methylated by an *O*-methyltransferase present in the leaf protein extract. In the presence of [$^{14}CH_3$]AdoMet, radiolabeled 16-methoxytabersonine is produced. Reactions included 0.5 mg of crude protein extract, 18 μM [$^{14}CH_3$]AdoMet, 4 mM DTT, 100 mM Tris-Cl (pH 8), and, optionally, 30 μM tabersonine, 1 mM NADPH, 1 mM NADH, or a combination of these substrates in a reaction mixture with a final volume of 100 μL. After the samples were incubated for 20 min at 30°C, the reactions were stopped with base, and radioactive alkaloids were separated from unreacted [$^{14}CH_3$]AdoMet with EtOAc. Alkaloids extracted from the reaction mixture were separated by TLC and subjected to autoradiography. Left, With solvent EtOAc-MeOH (9:1), tabersonine and labeled 16-methoxytabersonine (R_F 0.65) co-migrated. Right, With solvent hexane-diethylether (1:1), tabersonine (R_F 0.36) and 16-methoxytabersonine (R_F 0.25) were separated. Positions of alkaloid standards are shown in the margin. Components of the reaction mixture are indicated below the autoradiogram. AdoHcy, *S*-Adenosyl-L-homocysteine.

trace amounts of 16-[^{14}C]methoxytabersonine (Fig. 2, lane 1). These results suggest that trace amounts of 16-hydroxytabersonine must be present in the protein extract and were probably being methylated. Treatment of the extract with XAD-4 resin or Bio-beads (Bio-Rad) to remove small hydrophobic molecules did not reduce the production of 16-[^{14}C]methoxytabersonine. Addition of tabersonine (Fig. 2, lane 2) to the assay mixture did not increase product formation, whereas the addition of both NADPH and tabersonine resulted in the enzymatic synthesis of a radioactive product with an R_F value corresponding to that of 16-methoxytabersonine (Fig. 2, lane 3). Chromatography of the reaction mixture in a different solvent system (solvent B), which separated tabersonine from 16-methoxytabersonine, showed that the radioactive product migrated with an R_F corresponding to 16-methoxytabersonine (Fig. 2, lane 5). When the radioactive product was extracted from the TLC plate and analyzed by HPLC, the radioactivity eluted as a single peak and had a UV spectrum identical with that of 16-methoxytabersonine (Pyuskyulev et al., 1967).

Although NADH could replace NADPH as a substrate, the amount of product formed was only 20% of that obtained with NADPH (Fig. 2, lane 4). 16-OH activity was also shown to require molecular oxygen, since its removal from the reaction completely abolished the formation of the 16-methoxytabersonine (Table I). Furthermore, the addition to the reaction mixture of increasing concentrations of CO, a well-known competitive inhibitor of Cyt P-450-dependent monooxygenases, inhibited product formation in a concentration-dependent manner (Table I).

To determine whether the 16-hydroxylase is a microsomal enzyme, microsomal and soluble protein fractions were tested for enzyme activity. When soluble or microsomal protein fractions were incubated separately with NADPH, tabersonine, and [$^{14}CH_3$]AdoMet, small amounts of product were detected, whereas about 10 times more product was formed when enzyme assays were performed in the presence of both protein fractions (Table II). The apparent requirement of both membrane-bound and soluble proteins for product formation suggested that the 16-hydroxylation and the *O*-methylation reactions occurred in different cellular compartments.

To identify the cellular location of the *O*-methyltransferase, substrate for this reaction was synthesized enzymatically by incubating crude leaf protein extract with tabersonine and NADPH in the presence of oxygen. The alkaloid product, containing 16-OH-tabersonine, was extracted with EtOAc and the solvent was reduced to dryness. When increasing amounts of alkaloid product were incubated with [$^{14}CH_3$]AdoMet and soluble proteins, 16-[^{14}C]methoxytabersonine was produced in a concentration-dependent manner, whereas reactions with microsomal fractions did not result in any product formation (Table III). These results demonstrate that the *O*-methyltransferase is a soluble protein and suggest that the 16-hydroxylase occurs in the microsomal fraction.

Table I. *O_2 dependence of 16-OH activity and inhibitory effect of CO*

Gas mixtures were balanced to 100% with N_2. The 100% enzyme activity represents 140 pmol s^{-1} g^{-1} protein.

Assay Condition	Enzyme Activity
	%
10% O_2 (dark)	100
0% O_2 (dark)	0
10/10% O_2/CO (dark)	100
10/50% O_2/CO (dark)	46
10/90% O_2/CO (dark)	23
10% O_2 (light)	15

Table II. *Protein fractions required for the conversion of tabersonine to 16-methoxytabersonine*

Reactions included tabersonine, NADPH, [$^{14}CH_3$]AdoMet, and fractions of young leaves and were incubated for 60 min. S_{100} and microsomal fraction (P_{100}) were prepared by centrifugation (100,000*g*, 60 min) of a post-mitochondrial supernatant (10,000*g*, 20 min). The amounts of soluble and microsomal proteins used in a 100-μL reaction mixture were 0.2 and 1 mg, respectively.

Fractions in Assay	16-[^{14}C]Methoxytabersonine
	pmol
S_{100}	4
P_{100}	3
S_{100} and P_{100}	31

Identification of the Hydroxylase as a Cyt P-450-Dependent Monooxygenase

A requirement for NADPH and molecular oxygen by a membrane-bound hydroxylase generally suggests that the enzyme belongs to the Cyt P-450 class of monooxygenases. Thus, the effect of various Cyt P-450 inhibitors on the conversion of tabersonine to 16-methoxytabersonine was tested. CO was an effective inhibitor of the 16-hydroxylase at concentrations of 50 and 90% in the dark (Table I).

Table III. *Protein fractions required for the conversion of 16-hydroxytabersonine to 16-methoxytabersonine*

Reactions were incubated at 30°C for 60 min with S_{100} or microsomal fraction (P_{100}). After incubation, the product was purified by TLC and quantified by liquid scintillation chromatography.

Components in Reaction[a]			16-[^{14}C]-Methoxytabersonine
16-OH	[$^{14}CH_3$]AdoMet	Protein fraction	
μL	μCi		*pmol*
0	0.1	S_{100}	2
2	0.1	S_{100}	28
5	0.1	S_{100}	50
10	0.1	S_{100}	78
20	0.1	S_{100}	103
20	0.1	P_{100}	3

[a] 16-Hydroxytabersonine was prepared enzymatically from tabersonine. For that, tabersonine (120 nmol), NADPH (1 μmol), and desalted leaf proteins (6 mg) were incubated in 1 mL for 60 min. After basification, the reaction mixture was extracted with EtOAc and dried. The alkaloids were dissolved in 40 μL of MeOH and used for the *O*-methyltransferase assay.

Oxidized Cyt *c* displayed an IC_{50} of 1 μM (Table IV) and completely inhibited the 16-OH at a concentration of only 5 μM. Clotrimazole and miconazole with IC_{50}s of 50 and 300 μM were also effective inhibitors of 16-OH, whereas the general Cyt P-450 inhibitors 1-phenylimidazole and piperonylbutoxide were effective only in the millimolar range. Tetcyclasis, a powerfull inhibitor of *ent*-kaurene synthase was only effective at concentrations higher than 0.5 mM. Potassium cyanide (1 mM) and sodium azide (1 mM) did not inhibit the 16-OH at high concentration, which is characteristic of Cyt P-450 monooxygenases (Oshino et al., 1966).

Subcellular Localization

To investigate the subcellular localization of 16-OH, leaf protoplasts were lysed and cellular components were fractionated on a linear Suc gradient. The marker enzymes CCR, Cyt *c* oxidase, Chl, and malate dehydrogenase peaked at the expected densities of 1.11, 1.18, 1.16, 1.21, and 1.24 g/mL (Fig. 3), corresponding to ER, mitochondria, broken and intact chloroplasts, and microbodies, respectively (De Luca and Cutler, 1987). The activity of 16-OH in the gradient was nearly identical with that of the CCR pattern, the marker for ER (Fig. 3). Isolation and fractionation of protoplasts in the presence of 3 mM $MgCl_2$ resulted in a shifting of a significant fraction of 16-OH and CCR activities to higher densities (1.16–1.19 g/mL) (results not shown). These data suggest that the 16-OH is associated with the ER.

Properties of 16-OH

16-OH exhibited abnormal Michaelis-Menten kinetics for tabersonine, since concentrations greater than 30 μM were inhibitory. The apparent K_m for tabersonine was determined to be 11 μM (Fig. 4A). In contrast, the enzyme displayed normal Michaelis-Menten kinetics for NADPH, with an apparent K_m of 14 μM (Fig. 4B). The coupled enzyme assay catalyzing the conversion of tabersonine to 16-methoxytabersonine showed a pH optimum of 7.5 in phosphate buffer and of 8.0 in Tris-HCl (results not shown). The reactions were linear for 20 min at 30°C under the assay conditions used.

Table IV. *Effect of inhibitors of Cyt P-450 enzymes on 16-OH*

The 100% enzyme activity represents 340 pmol s^{-1} g^{-1} protein.

Inhibitors	IC_{50}
	μM
None	
Cyt *c*	1
Clotrimazole	50
Miconazole	300
Tricliphane	500
Flusilazole	>500
Tetcyclasis	>500
Piperonylbutoxide	2,000
1-Phenylimidazole	2,000

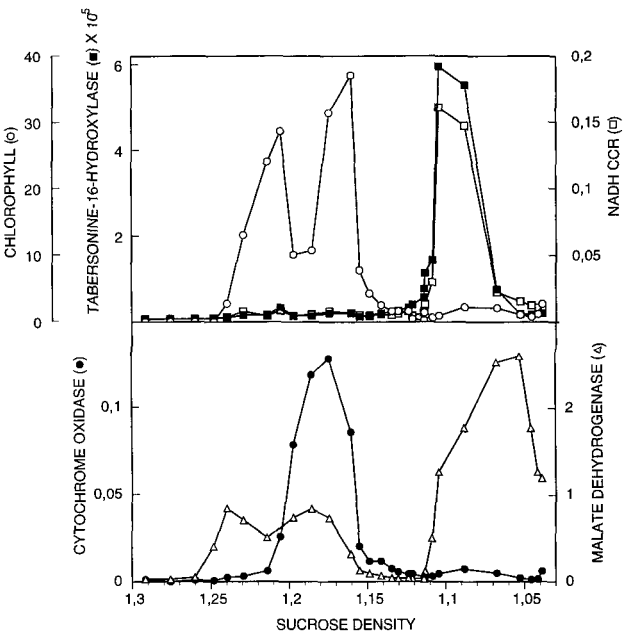

Figure 3. Linear Suc density gradient centrifugation of lysed protoplast fraction isolated from young *C. roseus* leaves. All enzyme activities are expressed as μmol substrate converted per min per mL fraction. Chl is expressed as μg per fraction.

Distribution and Expression of 16-OH Activity in *C. roseus*

The expression of the 16-hydroxylase during seedling development was investigated. *C. roseus* seeds, which were germinated and grown in the absence of light, showed a transient and low level of activity that peaked after 8 d of seedling development (Fig. 5). Exposure of seedlings to light 6 d after imbibition resulted in a 6-fold increase of 16-OH activity over dark-grown plants, which peaked after 9 d of seedling development and subsequently declined (Fig. 5).

In mature plants, the activity of 16-OH was abundant in young leaves (Fig. 6). However, the hydroxylase activity was about 50- to 100-fold lower in flower buds and roots, respectively, and absent in stems and old leaves. Cell-suspension cultures of *C. roseus* express 16-OH and *O*-methyltransferase activities, but the level was only about 20% of that in young leaves (Fig. 6).

DISCUSSION

The biosynthesis of vindoline from tabersonine in *C. roseus* has been suggested to include three enzymatic hydroxylations. The only one of these hydroxylations so far characterized is the second to last step, which is catalyzed by a 2-oxoglutarate-dependent dioxygenase (De Carolis et al., 1990; De Carolis and De Luca, 1993). The enzyme described here catalyzes the hydroxylation at C-16 of tabersonine and represents the first step in the conversion of tabersonine to vindoline. This novel catalytic activity was characterized using a sensitive, coupled enzyme assay. Leaf protein extracts were incubated with tabersonine and various cofactors known to be required by hydroxylases.

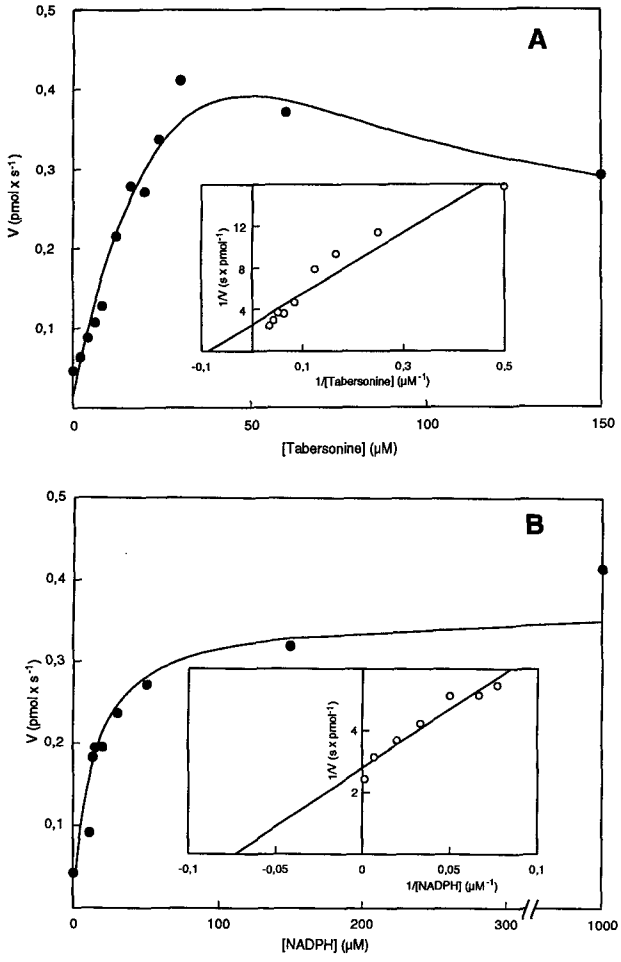

Figure 4. The effect of tabersonine (A) and NADPH (B) concentrations on the 16-OH activity. The inserts show the double-reciprocal plot. K_m = 11 μM for tabersonine and 14 μM for NADPH.

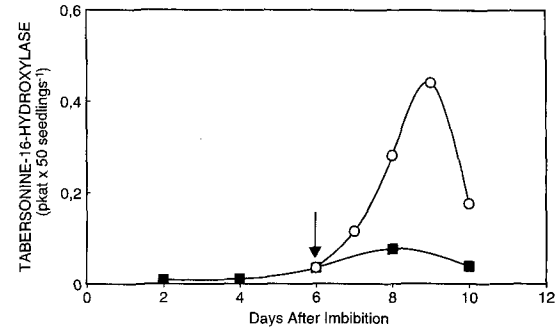

Figure 5. Induction of 16-OH activity in developing seedlings of *C. roseus*. The time course for 16-OH activity was performed with etiolated seedlings (■) and with etiolated seedling subjected to light treatment 6 d after imbibition (○). The enzyme assay for 16-hydroxylase was supplemented with leaf S_{100} as a source of *O*-methyltransferase activity.

The reaction product, 16-hydroxytabersonine, was detected and quantified by enzymatically converting it to ^{14}C-labeled 16-methoxytabersonine. The second reaction of the coupled assay was performed with a soluble *O*-methyltransferase (Fahn et al., 1985b) that catalyzes transfer of the methyl group from [$^{14}CH_3$]AdoMet to 16-hydroxytabersonine. Radioactively labeled 16-methoxytabersonine was then purified by TLC and quantified. To ensure conversion of 16-hydroxytabersonine to 16-methoxytabersonine, the S_{100} from immature leaves of *C. roseus* was added in excess to the 16-OH assay when tested in extracts that might lack *O*-methyltransferase activity (Figs. 3, 5, and 6). This fraction is a convenient source of *O*-methyltransferase activity (Table III) that is devoid of 16-OH activity (Table II).

16-OH has an absolute requirement for oxygen (Table I) and NADPH (Fig. 2). Furthermore, its association with the ER membrane (Fig. 3) and reduced activity in the presence of CO (Table I), Cyt *c*, and various P-450-specific inhibitors (Table IV) strongly suggests that 16-OH is a Cyt P-450-dependent monooxygenase.

This class of enzymes has been implicated in a variety of oxidative reactions in animals and plants. In mammals, they are involved in detoxification of drugs and other xenobiotics as well as in the metabolism of fatty acids, prostaglandins, and steroids (White and Coons, 1980; Nebert and Gonzalez, 1987). In plants, Cyt P-450 enzymes participate in the biosynthesis of alkaloids, fatty acids, flavonoids, GA, polyphenolic acids, pterocarpans, steroids, and terpenes, as well as in the detoxification of herbicides (reviewed by Donaldson and Luster, 1991; Durst, 1991; Sandermann, 1992). In *C. roseus*, for example, the Cyt P-450-dependent geraniol 10-hydroxylase involved in the biosynthesis of secologanin channels geraniol into the production of secologanin and subsequently into indole alkaloids (Meehan and Coscia, 1973). The 16-OH reported here is a Cyt P-450 monooxygenase also involved in indole alkaloid biosynthesis.

Microsomal monooxygenases are composed of two polypeptides: NADPH:Cyt P-450 reductase and Cyt P-450

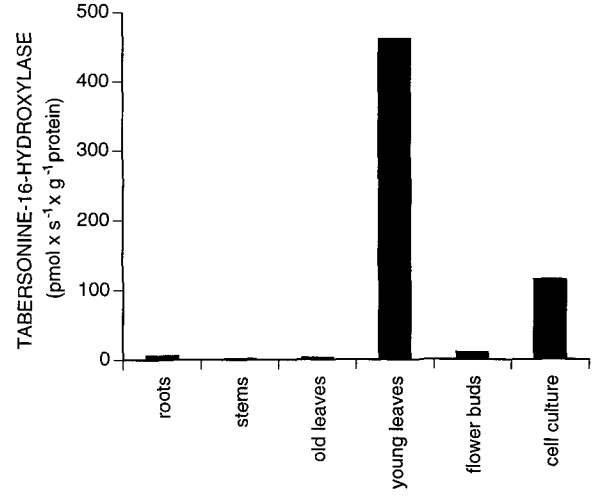

Figure 6. Distribution of 16-OH activity in *C. roseus* plant and in cell-suspension culture No. 615. Enzyme assay for 16-hydroxylase in plant extracts was supplemented with leaf S_{100} as a source of *O*-methyltransferase activity. The assay of 16-OH in cell culture extracts did not contain S_{100}.

(Donaldson and Luster, 1991). NADPH:Cyt P-450 reductase transfers electrons from NADPH to Cyt P-450. In vitro, NADPH:Cyt P-450 reductase can also transfer electrons to Cyt *c*, and thus addition of Cyt *c* can inhibit the Cyt P-450-dependent reaction. The involvement of NADPH: Cyt P-450 reductase in the 16-OH reaction was suggested by its requirement for NADPH and its inhibition by Cyt *c*. NADH:Cyt b_5 reductase and Cyt b_5 are also integral membrane proteins of microsomes that can reduce Cyt P-450 in animals (Taniguchi et al., 1984) and probably in plants (Donaldson and Luster, 1991). This may explain the ability of NADH to support the 16-OH reaction at reduced efficiency (Fig. 2).

Eukaryotic Cyt P-450-dependent enzymes are membrane-bound hemoproteins in which the heme moiety binds the co-substrate oxygen (White and Coon, 1980). 16-OH activity demonstrated an absolute requirement for oxygen (Table I). CO can also be bound by the heme moiety and inhibit the activity of Cyt P-450. In addition, CO inhibition of Cyt P-450-catalyzed reactions is photoreversible (Estabrook et al., 1963). CO inhibited 16-OH (Table I) in the range observed for other Cyt P-450-dependent reactions in plants (Karp et al., 1990; Funk and Croteau, 1993; Gerardy and Zenk, 1993; Rueffer and Zenk, 1994). However, the photoreversibility of this inhibition could not be tested by exposure of the reaction mixture to white light or to light filtered through a 10% $CuSO_4$ solution, which transmits light around 450 nm. Either treatment inhibited the accumulation of 16-methoxytabersonine (Table I), since it appeared to be unstable in presence of light (data not shown).

Cyt P-450 exists in various forms, which confer substrate specificity. Hepatic and insect Cyt P-450 that are involved in detoxification of exogenous chemicals possess a broad and overlapping substrate specificity (Ruckpaul and Rein, 1984; Ortiz de Montellano, 1986). In contrast, most Cyt P-450s from plants exhibit a narrow substrate specificity (Donaldson and Luster, 1991; Durst, 1991; Sandermann, 1992). Inhibitors can be used to block selectively specific Cyt P-450-catalyzed reactions and thus to distinguish between different forms of the enzyme (Donaldson and Luster, 1991). Tetcyclasis, a potent inhibitor of the Cyt P-450 *ent*-kaurene oxidase involved in GA biosynthesis (Rademacher, 1991), was a poor inhibitor of 16-OH (Table IV). However, the *N*-substituted imidazoles clotrimazole and miconazole, which efficiently inhibit C-6 and C-3 hydroxylation of the monoterpenoid limonene (Karp et al., 1990), were effective inhibitors of 16-OH in the micromolar range (Table IV). It is interesting that the terpenoid hydroxylases were not sensitive to ancymidol, another potent inhibitor of *ent*-kaurene oxidation (Karp et al., 1990). It appears that the Cyt P-450s that hydroxylate tabersonine and limonene share common properties and differ somewhat from the diterpenoid hydroxylase *ent*-kaurene oxidase.

It has previously been shown that enzymes involved in indole alkaloid biosynthesis in *C. roseus* seedlings are under strict light, developmental, and tissue-specific control. For example, enzymes involved in the early stages of indole alkaloid biosynthesis (Trp decarboxylase, strictosidine synthase) exhibit peak activity after 5 d of seedling development and are not stimulated by light (De Luca et al., 1986, 1988). In contrast, enzymes involved in the late stage of vindoline biosynthesis (NMT, 4-OH, DAT) reach a maximum activity 6 d after imbibition (De Luca et al., 1986, 1988; De Carolis et al., 1990). Furthermore, the 4-OH and DAT activities were stimulated 6- and 10-fold, respectively, when seedlings were treated with light (De Carolis et al., 1990; De Luca et al., 1988). The effect of light on 4-OH and DAT activities appears to be mediated by phytochrome (Aerts and De Luca, 1992; De Carolis, 1994). The transfer of seedlings to light also increased the activity of 16-OH 6-fold (Fig. 5). The 16-hydroxylase activity reached a maximum at d 9 of seedling development (Fig. 5). Thus, the first and the last two steps that convert tabersonine into vindoline are regulated by light. So far, NMT is the only known enzyme activity of this part of the pathway that is not activated by light (De Luca et al., 1988).

Enzymes of the late stages of vindoline biosynthesis display an organ-specific distribution in both seedlings and mature plants. NMT and DAT activities are highest in cotyledons but are absent from the roots of seedlings (De Luca et al., 1988). In mature plants, DAT shows a decreasing gradient of activity from the first to fourth leaf pairs (De Luca et al., 1985). Similarly, 4-OH activity decreases from the first to the fourth leaf pairs and is absent from older leaves and from roots (De Carolis, 1994; F. Vasquez-Flotta, personal communication). Consistent with its role as the first committed step in vindoline biosynthesis, 16-OH activity is most abundant in young leaves of *C. roseus* plants (Fig. 6). Therefore, tabersonine 16-hydroxylase, NMT, 4-OH, and DAT appear to be coordinately regulated in mature plants. In contrast, activities for 16-hydroxylase and *O*-methyltransferase (Fig. 6) but not NMT, 4-OH, and DAT (De Luca et al., 1987; F. Vasquez-Flotta, personal communication) were present in *C. roseus* cell-suspension cultures. Indole alkaloids with a C-16 methoxy functional group have been reported from *C. roseus* suspension cultures (Kutney et al., 1980a, 1980b; Stöckigt and Soll, 1980). The absence of NMT, DAT, and 4-OH activities in suspension cultures indicates a degree of uncoupling in the regulation of the first two and the last four biosynthetic steps involved in the conversion of tabersonine to vindoline.

A complex model of vindoline biosynthesis has evolved with respect to enzyme compartmentation and metabolic transport mechanisms. For example, two early steps in indole alkaloid biosynthesis catalyzed by strictosidine synthase and geraniol 10-hydroxylase are localized within specialized vesicles and their membranes, respectively (Madyastha et al., 1977; McKnight et al., 1991). Other enzymes involved in early (Trp decarboxylase) and late (DAT) stages of vindoline biosynthesis appear to occur in the cytosol, whereas NMT is a thylakoid-associated enzyme (De Luca and Cutler, 1987). The localization of 16-OH to the ER (Fig. 3) adds to the complexity of the compartmentation of this biosynthetic pathway. Studies of animal systems indicate that ER-associated Cyt P-450-dependent enzymes are integral membrane proteins anchored by an N-terminal signal peptide and that their active domain is located in the cytosol (Black, 1992). The topology of plant

Cyt P-450s is not known but the similarity of plant and animal Cyt P-450 genes, including regions encoding both the hydrophobic N-terminal and heme-binding domains, suggests a conserved topography (Bozak et al., 1990; Teutsche et al., 1992; Vetter et al., 1992; Meijer et al., 1993). Based on these findings we propose that the 16-hydroxylation of tabersonine probably also occurs on the cytosolic side of the ER membrane.

In summary, the data reported here strongly suggest that 16-OH activity consists of an ER-associated Cyt P-450 monooxygenase. This enzymatic activity shows a tissue-specific, development-specific, and light-regulated expression similar to the last two enzymatic steps of vindoline biosynthesis. However, the presence of this enzyme as well as the *O*-methyltransferase in *C. roseus* cell cultures contrasts with the absence of the last three steps in vindoline biosynthesis. These results further define the enzymatic activities whose presence would be required to permit vindoline biosynthesis in cell cultures.

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