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Jasmonate-induced epoxidation of tabersonine by a cytochrome P-450 in hairy root cultures of *Catharanthus roseus*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Methyl jasmonate, a chemical inducer of secondary metabolism, was shown to promote tabersonine **2** biosynthesis in hairy root cultures of *Catharanthus roseus*. Tabersonine 6,7-epoxidase activity was detected in total protein extract of jasmonate-induced hairy root cultures using labeled ^{14}C-tabersonine **2**. This enzyme converted tabersonine **2** to lochnericine **3** by selective epoxidation at positions 6 and 7 via a reaction dependent on NADPH and molecular oxygen. Carbon monoxide, clotrimazole, miconazole, and cytochrome C were shown to be strong inhibitors of the enzyme. The activity was found in microsomes, indicating that tabersonine 6,7-epoxidase was a cytochrome P-450-dependent monooxygenase.

Keywords: *Catharanthus roseus*; Apocynaceae; Tabersonine; Lochnericine; Hörhammericine; Hairy roots; Methyl jasmonate; Epoxidation; Cytochrome P-450

1. Introduction

Alternative means to produce vinblastine and vincristine, two major anticancer alkaloids isolated from *Catharanthus roseus*, have been extensively investigated during the last 20 years. Despite intense efforts, plant cell, tissue, and hairy root cultures have failed to be useful sources for these two bisindole alkaloids that are composed of catharanthine and vindoline (**1**) monomers. Nevertheless, real progress was achieved when certain cell lines and selected hairy root lines were shown to produce and accumulate catharanthine and precursors of vindoline **1**, such as tabersonine **2**. Concurrently, many studies were devoted to the enzymology and regulation of the biosynthesis of these monomers. The latter investigations led to a better understanding of the pathways leading to indole alkaloids in *C. roseus*, and were essential steps for the development of new strategies for their production (Meijer et al., 1993).

Unlike unstable cell cultures, hairy roots have been shown to consistently produce and accumulate particular alkaloids (Bhadra et al., 1993). The advantages of hairy roots for the manufacture of alkaloids have been attributed to their high level of differentiation, together with their apparent genetic stability and to the fact that they can be readily transformed. Therefore, many studies were focused on hairy root cultures with the aim of increasing the production yields of indole alkaloids (Bhadra et al., 1993). While tabersonine **2** is converted to vindoline **1** by six enzymatic steps in the aerial part of the plant (Fig. 1; De Luca and Laflamme, 2001; De Luca et al., 1998; Meijer et al., 1993), another biosynthetic pathway occurs in hairy roots (Fig. 1). Indeed, the

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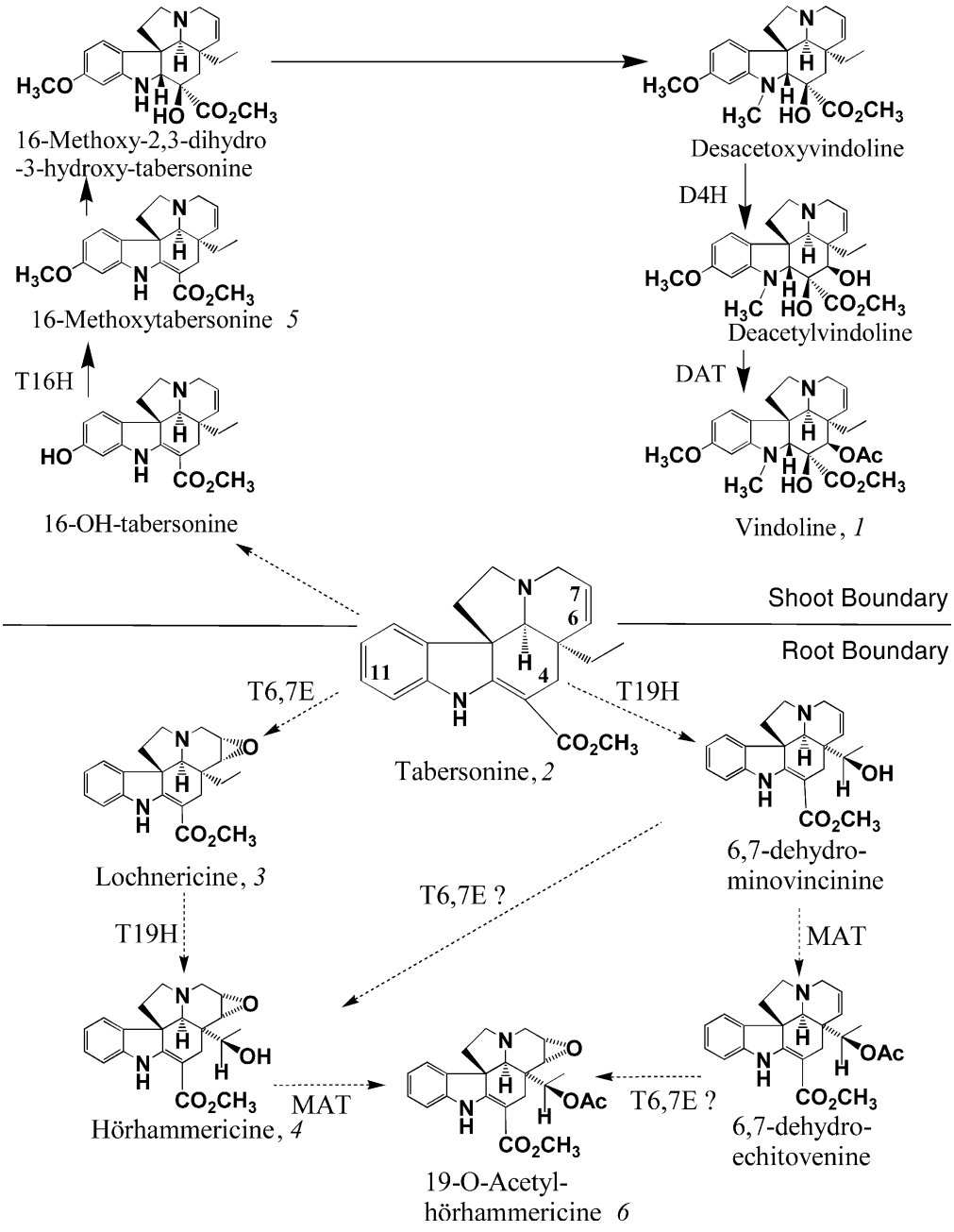

Fig. 1. Principal secondary metabolites derived from tabersonine in aerial parts and in hairy root culture of *C. roseus*. In the aerial parts, tabersonine **2** is converted to vindoline **1** by six enzymatic steps. In roots the biosynthetic pathway leading to hörhammericine **4** involves several different possible conversions that have not been well characterized. The numbering system used was as for aspidospermidine alkaloids in *Chemical Abstracts* (1987–1991).

accumulation of lochnericine **3** and hörhammericine **4**, two oxygenated derivatives of tabersonine **2**, has been shown to occur in hairy root cultures (Shanks et al., 1998). Lochnericine **3** and hörhammericine **4** both possess an epoxide function at positions 6 and 7, instead of the double bond found in tabersonine **2**. As seen in Fig. 1, production of these epoxide derivatives of tabersonine **2** provides a competitive pathway that may prevent the conversion of tabersonine **2** to intermediates for the biosynthesis of vindoline **1**, by diverting away the flux of tabersonine **2**. It is presently unclear how vindoline **1** biosynthesis can take place when the pathway for tabersonine **2** biosynthesis in the roots is spatially separate from several of the terminal steps in vindoline **1** biosynthesis that occur in specialized idioblasts and laticifers in above ground plant parts (St Pierre et al., 1999). A global metabolic engineering strategy for vindoline **1** production therefore requires a better understanding of tabersonine **2** biosynthesis and metabolism.

Recently, tabersonine **2** metabolism in hairy root cultures of *C. roseus* was shown to be highly sensitive to jasmonic acid (Morgan and Shanks, 1999). The effect of jasmonic acid application on the accumulation of hörhammericine **4** and lochnericine **3** in the tissues has also been quantified (Rijhwani and Shanks, 1998). These studies showed a significant increase in hörhammericine **4** and lochnericine **3** production with jasmonic acid treatment, while tabersonine **2** accumulation was decreased. The jasmonate-activated transformation of tabersonine **2** to lochnericine **3** and hörhammericine **4** has been studied in hairy root cultures with inhibitors of oxygenase reactions (Morgan and Shanks, 1999), but to date, the enzymes involved in these oxidations have yet to be characterized. This report describes a jasmonate-inducible tabersonine **2**-6,7-epoxidase that is responsible for the conversion of tabersonine **2** to lochnericine **3**, and shows it to be a microsomal Cyt P-450-dependent monooxygenase.

2. Results

2.1. Enzymatic epoxidation of tabersonine

An enzyme assay was developed to study the conversion of tabersonine **2** into lochnericine **3** in the presence of a total desalted protein extract from hairy root cultures of *C. roseus*. Since the desalted extract still contained tabersonine **2** and other derivatives, the assay was performed with [methyl-^{14}C]-tabersonine **2** as substrate in order to detect specific radioactive products independently of alkaloid products already present in the extract. TLC [developed with petroleum ether–EtOAc (1:1)] of [methyl-^{14}C]-tabersonine **2** gave an intense radioactive spot with an $R_f = 0.53$ (Fig. 2, lane 1). Incubation of the crude desalted protein extract with radioactive tabersonine **2** and NADPH produced a new radioactive compound with an R_f value of 0.37 that corresponds to the R_f of lochnericine **3** standard (Fig. 2, lane 2). As shown below, this product was also identified by LC/UV analysis using photodiode array detection and by comparison with an authentic standard. The low specific activity of the substrate (3.1 μCi /μM) and significant amounts of unlabeled alkaloids, including tabersonine **2** and lochnericine **3** (Fig. 3A), in the extract gave a relatively low incorporation of the radioactive tabersonine **2** into lochnericine **3** (Fig. 3B and C). Therefore, a large amount of unconverted ^{14}C-tabersonine **2** was observed (Fig. 3B and C). Production of radiolabeled lochnericine **3** was not observed in the assay in the absence of NADPH (Fig. 2, lane 3), but a new radioactive compound with R_f values of 0.12 close to that of hörhammericine **4** ($R_{f=}0.09$) was produced, together with a very polar product that did not migrate in this solvent system. The two latter metabolites were not further identified. NADH could partially replace NADPH as a co-substrate, since the amount of lochnericine **3** formed was only 30% of that obtained with NADPH (Fig. 2, lane 4). In addition, the other two slowly migrating products were also formed in the presence of NADH. A negative control was performed with denatured enzyme (5 min in boiling water) to avoid any confusion with non-enzymatic reactions (Fig. 2, lane 5).

2.2. Product analysis

The major radioactive product extracted from standard NADPH-containing assays appeared to be lochnericine **3** as it co-migrated with lochnericine **3** standard on TLCs when chromatographed in three different solvent systems (systems I–III, see Experimental). In order to confirm the identification by spectroscopic evidence, alkaloid extracts were analysed by HPLC/DAD-UV before and after the enzymatic assay. The chromatogram exhibited in Fig. 3A shows the alkaloid content of the crude protein extract before incubation with radiolabeled substrate (EtOAc extract). The UV trace was recorded at 330 nm, corresponding to the UV maxima of both tabersonine **2** and lochnericine **3** in their UV spectra. Lochnericine (**3**) was shown to be the major alkaloid present in the extract. The presence of smaller amounts of endogenous tabersonine **2** (**1**) was also observed. Alkaloids x and y were not identified. The alkaloid profile after enzymatic incubation (Fig. 3B) exhibited a larger peak corresponding to tabersonine **2**, representing the low specific activity (3.1 μCi/μM) mixed radiolabelled and unlabelled tabersonine **2** substrate that was added to the assay. As a consequence of the enzymatic reaction, a slight increase of the peak 2 corresponding to lochnericine **3** was also observed.

In order to demonstrate that lochnericine **3** was synthesized from tabersonine **2**, fractions were collected every 30 s at the outlet of the photodiode array detector and radioactivity was detected by liquid scintillation spectrometry. The reconstructed radioactive trace presented in Fig. 3C revealed a radioactive peak corresponding to [^{14}C]-lochnericine **3** resulting from an enzymatic epoxidation of [^{14}C]-tabersonine **2**.

2.3. Induction with methyl jasmonate

Enzyme assays for tabersonine epoxidase clearly showed that methyl jasmonate treatment of hairy root cultures significantly activated tabersonine **2** epoxidase activity. Methyl jasmonate treatment increased detectable tabersonine **2** epoxidase activity 6-fold within after 48 h of treatment compared to untreated control cultures (Fig. 4). For this study, enzymatic assays were also performed in the presence of KCN to avoid any competitive oxidation reactions that might occur as a result of non-specific peroxidase activities. This result strongly

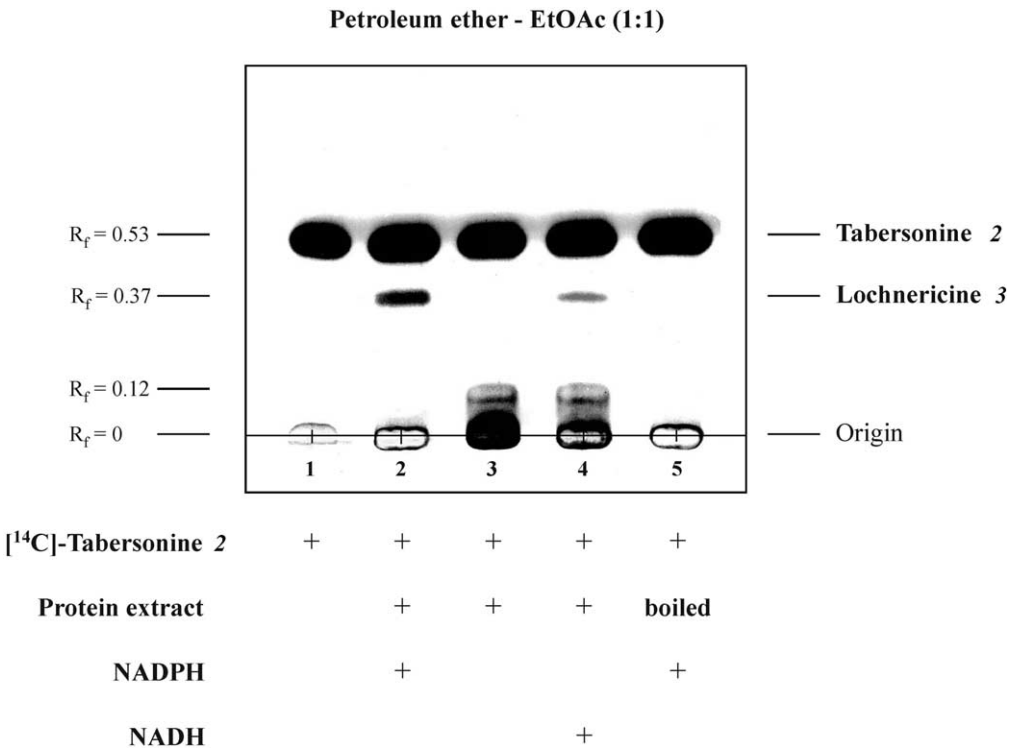

Fig. 2. Co-factor and substrate requirements for tabersonine 6,7-epoxidase. The reactions included 28 μM tabersonine **2** (35,000 *dpm* [^{14}C]-tabersonine **2**), 0.4 mg of crude protein extract (boiled for lane 5), 4 mM DTT, 14 mM β-mercaptoethanol, 100 mM Tris–HCl (pH 7.8), and optionally, 1 mM NADPH or 1 mM NADH, in a final volume of 100 μl. The samples were incubated for 20 min at 30 °C and the reactions were stopped with NaOH. The alkaloids were extracted with EtOAc, separated on TLC and subjected to autoradiography. Unconverted [^{14}C]-tabersonine **2** presented an R_f of 0.53, while the enzymatic product, lochnericine **3**, was observed at $R_f = 0.37$ under certain reaction conditions. Unidentified polar compounds could also be detected ($R_f = 0.12$) under different reaction conditions.

suggested that the presence of methyl jasmonate promoted tabersonine **2** epoxidase activity in hairy root cultures. A slight effect due to the addition of methanol was also observed, when compared with an untreated batch (Fig. 4, 48 h without induction).

2.4. Identification of the tabersonine 2 epoxidase as a Cyt P-450 monooxygenase

Previous studies have suggested that cytochrome P-450 monooxygenases might be involved in the conversion of tabersonine **2** to lochnericine **3** and hörhammericine **4**, since inhibitors of this type of enzyme reduced the accumulation of the two products in hairy roots (Morgan and Shanks, 1999). Various experiments were then performed in this study in order to demonstrate that T6,7P was a Cyt P-450-dependent monooxygenase.

Tabersonine **2** epoxidase requires molecular oxygen, since its removal from the reaction completely abolishes the formation of lochnericine **3** (Table 1). In addition, carbon monoxide, a well-known competitive inhibitor of Cyt P-450-dependent monooxygenases was tested. The results, presented in Table 1, clearly show the inhibition of lochnericine **3** production by CO in a concentration-dependent manner. The inhibition values observed for tabersonine epoxidase were very similar to those measured in young leaf extracts for tabersonine 16-hydroxylase (St Pierre and De Luca, 1995). The inhibitory effect of CO strongly suggested tabersonine epoxidase to be a Cyt P-450 monooxygenase.

In order to determine if tabersonine **2** epoxidase was a microsomal enzyme, microsomal (P_{100}) and soluble (S_{100}) protein fractions were prepared (see Experimental) and tested for enzyme activity. In presence of NADPH and [^{14}C]-tabersonine **2**, production of lochnericine **3** was only observed in microsomal fractions (results not shown). The addition of the S_{100} fraction to the latter assays did not influence the enzymatic activity or result in production of any further products. Tabersonine **2** epoxidase was thus demonstrated to be a membrane-bound protein.

In addition to carbon monoxide, different enzyme inhibitors were tested, some of which were general Cyt P-450 inhibitors. The IC_{50} values measured are listed in Table 2 and are compared to those reported for tabersonine 16-hydroxylase (St. Pierre and De Luca, 1995). The three Cyt P-450 inhibitors clotrimazole, miconazole, and cytochrome C were effective inhibitors of tabersonine **2** epoxidase in the micromolar range (IC_{50} values of 2, 2.5, and 4 μM, respectively). Tetcyclasis, a powerful inhibitor of *ent*-kaurane synthase exhibited an IC_{50} of 700 μM, while 1-aminobenzyltriazole did not

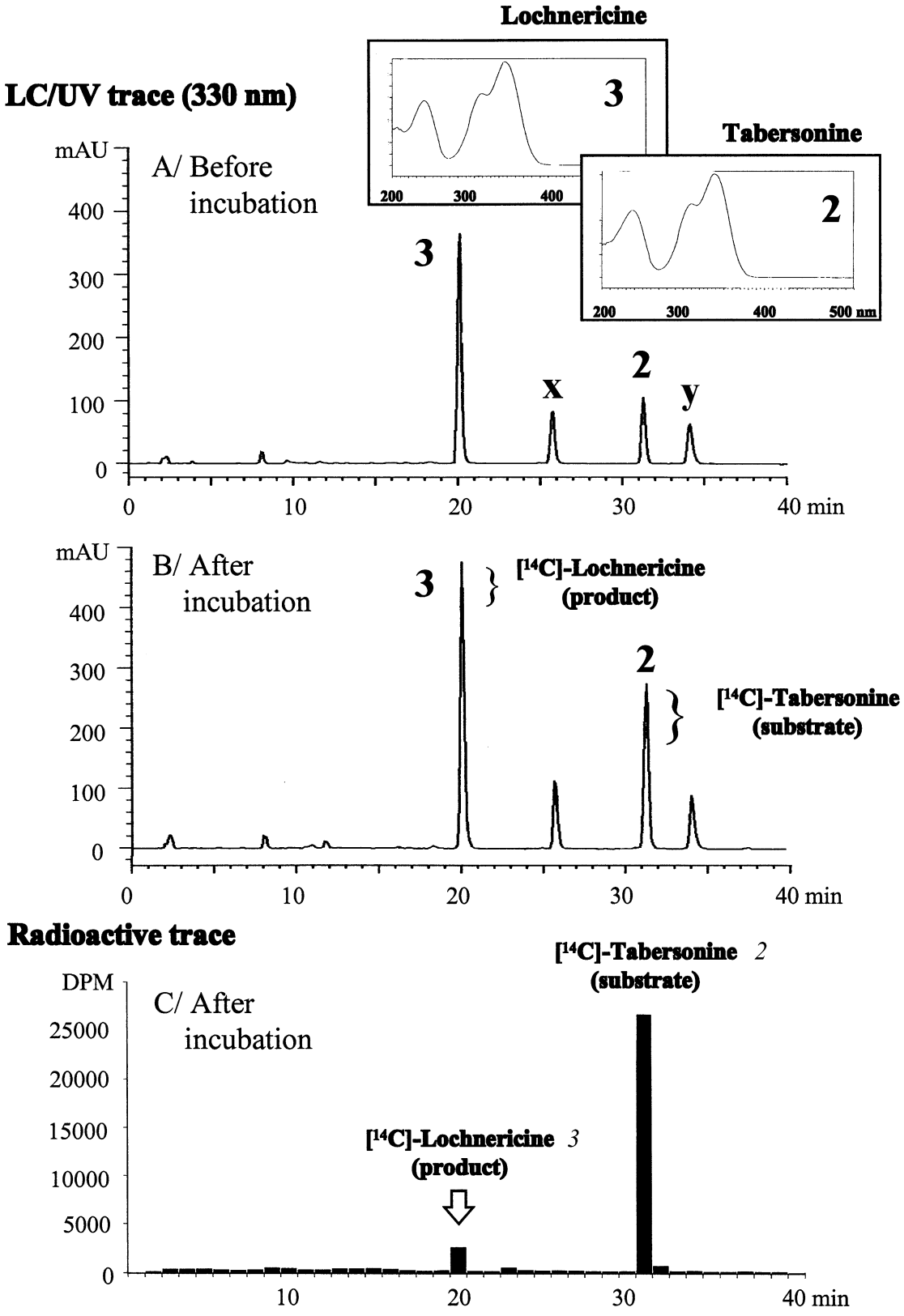

Fig. 3. LC/DAD-UV analyses of the alkaloid contents before and after the incubation with crude protein extract. Before the incubation (chromatogram A), the presence of four endogenous metabolites was observed, two of which have been identified as tabersonine (**2**) and lochnericine (**3**) as well as 2 uncharacterized non-radioactive compounds (x,y). The UV spectra recorded on-line of these two alkaloids are presented. After the incubation (chromatogram B), a larger peak corresponding to tabersonine **2** was visible, due to the presence of unconverted substrate. A slight increase of the peak **3**, resulting from the enzymatic production of lochnericine **3**, was observed. The chromatogram C represents the reconstructed radioactive trace after incubation. Peaks corresponding to unconverted [^{14}C]-tabersonine **2** and [^{14}C]-lochnericine **3** were observed.

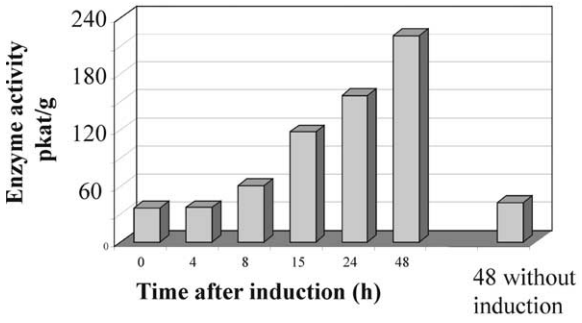

Fig. 4. Induction of tabersonine 6,7-epoxidase activity in hairy root cultures of *C. roseus* by methyl jasmonate. The time course shows a rapid increase of the enzymatic activity compared to the untreated sample (0 h) or the negative methanol treated control (48 h).

Table 1
O_2 dependence and inhibitory effect of carbon monoxide on tabersonine 6,7-epoxidase and tabersonine 16-hydroxylase[a]

Assay condition	Enzyme activity (%)	
	Tabersonine 6,7-epoxidase (in hairy roots)	Tabersonine 16-hydroxylase (in young leaves)
Open air	100	100
0% O_2	0	0
10% O_2	84	95
10% O_2/10% CO	82	n.d.
10% O_2/50% CO	57	n.d.
10% O_2/90% CO	26	35
100% CO	0	n.d.

n.d., not determined.

[a] Gas mixtures were balanced to 100% with N_2. Tabersonine 16-hydroxylase activity were measured as described by St. Pierre and De Luca (1995) with crude protein extract of young leaves of *C. roseus*. The 100% enzyme activity represents 260 and 340 pkat/g protein, respectively for Tabersonine 6,7-epoxidase and Tabersonine 16-hydroxylase.

Table 2
Effect of different inhibitors on tabersonine 6,7-epoxidase and tabersonine 16-hydroxylase[a]

Inhibitors	IC_{50} (μM)	
	Tabersonine 6,7-epoxidase (in hairy roots)	Tabersonine 16-hydroxylase (in young leaves)
Cytochrome C	4	1
Clotrimazole	2	50
Miconazole	2.5	300
1-Aminobenzotriazole	No inhibition	n.r.
Tetcyclasis	700	> 500
Potassium cyanide	2000	> 1000
Sodium azide	No inhibition	> 1000

[a] The 100% enzyme activity represents 370 and 340[b] pkat/g protein for Tabersonine 6,7-epoxidase and Tabersonine 16-hydroxylase, respectively.

[b] Values from St. Pierre and De Luca (1995); n.r., not reported.

significantly affect the production of lochnericine **3** in our enzyme assays. These results suggest that tabersonine 6,7-epoxidase is a Cyt P-450 monooxygenase. Moreover, potassium cyanide and sodium azide did not inhibit tabersonine epoxidase at high concentration (2 mM), which is also characteristic for Cyt P-450 monooxygenase (Oshino et al., 1966).

2.5. Properties of tabersonine 6,7-epoxidase

Kinetic studies showed that the enzyme has an apparent K_m for NADPH of 17 μM. Due to the presence of large amounts of endogenous substrate in the crude desalted protein extract (30 μM) and the low specific radioactivity of the labeled tabersonine **2**, the apparent K_m for tabersonine **2** could not be measured. Enzymatic assays also showed that labeled 16-$O^{14}CH_3$-tabersonine **5** was also accepted as a substrate by tabersonine epoxidase. However, the enzyme activity measured with 16-$O^{14}CH_3$-tabersonine **5** was 100 times lower than that obtained with tabersonine **2** as substrate. The product synthesized was presumed to be 16-$O^{14}CH_3$-lochnericine **3** (Furuya et al., 1992) due to its chromatographic behavior on TLC.

Tabersonine **2** epoxidase activity was not observed in cell cultures, young leaves, or in fresh roots of the mature plant. However, none of these tissues had been pre-treated with methyl jasmonate elicitor prior to extraction and enzyme assay. Very low level of tabersonine **2** epoxidase activity was found in roots of developing seedlings and unlike the results obtained with hairy roots (Fig. 4) methyl jasmonate treatment had no effect on the level of tabersonine **2** epoxidase in seedlings. However, these assays revealed that new, unidentified, polar radioactive products could be produced from [methyl-^{14}C]-tabersonine **2**.

3. Discussion

Extensive studies to quantitate the production of indole alkaloids in *C. roseus* hairy root cultures have revealed that they accumulate several alkaloids including ajmalicine, serpentine, catharanthine, tabersonine **2**, hörhammericine **4**, and lochnericine **3** (Shanks et al., 1998; Morgan and Shanks, 2000). The presence of tabersonine **2** in hairy roots has raised speculation that this intermediate in vindoline **1** biosynthesis, together with catharanthine, is transported from this potential site of biosynthesis through the vasculature to the stem and to the leaves where tabersonine **2** is further elaborated into vindoline **1** within laticifers and/or idioblasts (St. Pierre et al., 1999). However, oxidized derivatives of tabersonine **2**, such as hörhammericine **4** and lochnericine **3**, are present at 5–15 times the levels of tabersonine **2** in hairy roots (Shanks et al., 1998) and

presumably this prevents their transport and/or use for vindoline **1** biosynthesis. In this context, it would interesting to determine the rate of biosynthesis of tabersonine **2** to see what proportion of the intermediate is used for the manufacture of these oxidized end products. The pattern of accumulation of tabersonine **2**, lochnericine **3**, and hörhammericine **4** (Shanks et al., 1998) suggests that tabersonine **2** is first converted to its epoxide (lochnericine **3**) before further hydroxylation and *O*-acetylation (Laflamme et al., 2001) to form hörhammericine **4**. The alkaloids accumulating in hairy roots (Shanks et al., 1998) also suggest that the conversion of lochnericine **3** to 19-hydroxylochnericine **3** is probably a rate limiting step for the production of hörhammericine **4**.

The present studies show that microsomes of 10–14 day old hairy roots oxidized ^{14}C-tabersonine **2** into a new product in a reaction that required NADPH and O_2 (Table 1, Fig. 2). These results together with the ability of carbon monoxide (Table 1) and inhibitors of cytochrome P450s (Table 2) to inhibit this microsomal reaction strongly suggests that this oxidation is catalyzed by a cytochrome P450 dependent monooxygenase. This oxidized radioactive product was further characterized by thin layer chromatography and autoradiography to show that it co-migrated with lochnericine **3** (Fig. 2). Analysis of the product by high performance liquid chromatography coupled to DAD/UV spectral analysis also further confirms it to be lochnericine **3** (Fig. 3). This reaction catalyzed by the enzyme tabersonine **2**-6, 7-epoxidase (Fig. 1) may therefore be responsible for channeling tabersonine **2** into terminal hairy root products like hörhammericine **4** and 19-*O*-acetyl hörhammericine **6** (Shanks et al., 1998).

The previous studies of Morgan and Shanks (1999) showed that the oxygenase inhibitor, 1-aminobenzotriazole, specifically inhibited the formation of hörhammericine **4**, whereas clotrimazole inhibited the accumulation of lochnericine **3** in hairy root cultures. In addition, the use of jasmonic acid together with these inhibitors suggested that an inducible cytochrome P-450 enzyme was responsible responsible for the formation of hörhammericine **4**. The present study suggests that the jasmonate inducible enzyme is tabersonine 6,7 epoxidase.

4. Experimental

4.1. Chemicals

NADPH, NADH, dithiothreitol (DDT) and cytochrome C were purchased from Boehringer Mannheim. Clotrimazole and miconazole were from Sigma. 1-Aminobenzotriazole (ABT) was from Acros (NJ, USA) and methyl jasmonate was a gift from Firmenich (Geneva, Switzerland). Tetcyclasis was a gift from Dr. François Tardiff. Lochnericine **3** and hörhammericine **4** standards were a gift from Dr. Jacqueline V. Shanks. All other chemicals were of analytical grade.

4.2. Hairy root cultures and induction with methyl jasmonate

C. roseus hairy roots cultures (line J-1) were grown on sterile liquid media containing 3% (w/v) sucrose and Gamborg's B5/2 mineral salts and vitamins as described by Vazquez-Flota et al. (1994), and by Peraza-Sanchez et al. (1998). New cultures were initiated every 21 days by placing root tips in 250 ml flasks containing 100 ml of media, which were shaken at 130 rpm at 25 °C in the dark. Induction with methyl jasmonate was performed by adding 200 μl of a stock methanolic solution of methyl jasmonate (2: 1000, v/v) to each flask.

4.3. Seedling growth

Batches of *Catharanthus roseus* seeds (cv. Delicata) were sterilized for 1 min in 70% ethanol and washed several times in sterile water. Approximately 100 seeds were grown in 9 cm Petri dishes containing three layers of filter paper wetted with 3 ml sterile water. After the plates were sealed with Parafilm, the seeds were germinated and grown in the dark under controlled conditions for 4 days (25 °C, 70% RH). After 4 days of growth some batches of seeds were exposed to methyl jasmonate vapour diluted in MeOH, where 9 μl of this solution was placed in a tiny cup placed in the center of the Petri dish. The final Petri plate atmosphere would be 6 ppm of methyl jasmonate if all of it entered the gaseous phase. After 7 days of growth, some seedlings were kept in the dark and some were exposed to light. Samples were harvested at 4, 7, and 10 days of growth, frozen in liquid nitrogen, and kept at −80 °C until they were used for the enzyme assays.

4.4. Preparation of crude protein extract

Hairy root cultures (10–14 days old) were treated with methyl jasmonate (methanolic solution, 0.4 μl/flask) 24 h before sample collection (except for time course experiments). The plant material was ground in the presence of acid-washed sand and 1 ml/gfw of grinding buffer (100 mM Tris–HCl buffer, pH 7.8, 4 mM DTT and 14 mM β-mercaptoethanol) using a mortar and pestle. The slurry was centrifuged at 700 *g* for 5 min at 4 °C and the supernatant was desalted twice on a Sephadex G-25 PD-10 column (Amersham). The crude protein extract, containing between 0.3 and 0.6 mg protein/100 μl, was used directly for enzyme assays.

4.5. Microsomal and soluble protein fractions

Hairy root cultures (10–14 days old) were ground in liquid nitrogen. The frozen powder was mixed with 1

ml/gfw of grinding buffer (100 mM Tris–HCl, pH 7.8, 4 mM DTT and 14 mM β-mercaptoethanol), and the slurry was filtered through a 120 μm nylon filter. The filtrate was centrifuged at 10,000 *g* for 10 min at 4 °C in order to recover the supernatant that was then centrifuged at 100,000 *g* for 75 min at 4 °C. The 100,000 *g* supernatant represented the total soluble protein fraction (S_{100}), while the pellet contained the microsomal fraction (P_{100}) that was resuspended in extraction buffer.

4.6. Protein determination

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

4.7. Preparation of [methyl-^{14}C]-tabersonine (2)

2.7.1. Dilution of ^{14}C-methanol

A metal tube was inserted carefully into the neck of a break-seal vial containing ^{14}C-methanol (1.2 μl, 1 mCi) and the vial was sealed with a serum cap and Parafilm. The tip of the vial was cooled in an acetone/dry-ice bath to approximately −10 °C and the break-seal was broken by vigorous shaking of the metal tube. Dry, freshly distilled dichloromethane (1 ml) was injected into the vial and 10 μl of the resulting solution was removed and mixed immediately with scintillation cocktail. This sample was counted to indicate that a total of 290 μCi ^{14}C-methanol was contained in the 1 ml of dichloromethane solution. A small sample of the resulting product was removed and analyzed for purity by HPLC equipped with a radiodetector.

4.7.2. Preparation of [methyl-^{14}C]-tabersonine (2)

Tabersonine **2** was saponified by treatment with potassium hydroxide to afford, after work-up, tabersoninic acid. To a stirred solution of tabersoninic acid (10.0 mg, 31 μM) in dry dichloromethane (2.5 ml) contained in a flask sealed with a septum cap was added freshly distilled oxalyl chloride (6 μl, 62 μM). The resulting solution was stirred at room temperature for 20 min and then 290 μCi ^{14}C-methanol in dichloromethane (1 ml) was addded. After an additional 10 min, triethylamine (10 μl) was added and stirring continued for a further 5 min. A sample of the reaction mixture was removed and analysed by 500 MHz NMR, which revealed the presence of tabersonine **2** and triethylamine. *m*/*z* (APCI-ve) 279 (100%, M−1), 280 (32, M), 281 (5, M + 1).

4.8. Enzyme assay

The tabersonine 6,7-epoxidase (T6,7E) assay contained 10 μl NADPH (10 mM), 80 μl of crude protein extract, prepared as described above, and 28 μM [methyl-^{14}C]-tabersonine **2** (35,000 *dpm*) in a final volume of 100 μl. Methyl-^{14}C-tabersonine **2** was added as a methanolic solution and dried in vacuo immediately before the assay. After incubation for 20 min at 30 °C, the assay was terminated by the addition of 10 μl of 0.5 M NaOH. Alkaloids were extracted with EtOAc (2×500 μl). After vortexing for 2 min, the organic phase was separated from the aqueous phase by centrifugation at 10,000 *g* for 5 min. The EtOAc fractions were dried in vacuo, redissolved in 20 μl MeOH and subjected to HPLC (see below) or TLC (Kieselgel 60 F254, Merck) using petroleum ether–EtOAc (1:1, v/v) (system I), MeOH–EtOAc (1:9, v/v) (system II) or petroleum ether–diethylether (1:1 v/v) (system III) as mobile phase. The TLC plates were autoradiographed (Kodak XAR-5 film) or sprayed with ceric ammonium sulfate reagent (Farnsworth et al., 1964) in order to reveal the alkaloid compounds. The radioactive regions of the TLC plates corresponding to the different ^{14}C-labeled alkaloids were cut out and placed in scintillation cocktail. Quantification was performed by liquid scintillation spectrometry. The expression of enzymatic activity was corrected by subtracting the value obtained in a control assay performed with boiled enzyme.

4.9. LC/UV analysis

Reversed-phase HPLC analyses of the alkaloid extracts were carried out with a Waters 600E solvent delivery system, an on-line UV Waters 991 photodiode array detector and a Waters 717 autosampler. Separation of alkaloid substrate and product was achieved by chromatography of the reaction on a Macherey-Nagel Nucleosil RP-18 column (3 μm, 150 × 4.6 mm) using a MeCN–H_2O gradient (55:45 to 30:70) over 30 min followed by a 10 min wash under isocratic conditions (70% MeCN). The flow rate was 1 ml/min and 0.2% NH_4OAc was added to water to optimize pH conditions. The UV trace was observed at 330 nm and UV spectra were recorded between 190 and 500 nm.

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