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Inhibitor studies of tabersonine metabolism in *C. roseus* hairy roots

John A. Morgan [a], Jacqueline V. Shanks [a, b, *]

[a] *Rice University, Department of Chemical Engineering, MS-142, 6100 S. Main St., Houston, TX 77005-1892, U.S.A.*
[b] *Rice University, Department of Bioengineering, MS-142, 6100 S. Main St., Houston, TX 77005-1892, U.S.A.*

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Abstract

The conversion of tabersonine to lochnericine and hörhammericine was investigated in *C. roseus* hairy root cultures. The accumulation of lochnericine and hörhammericine, like tabersonine, was associated with growth. Through the use of oxygenase inhibitors, 1-aminobenzotriazole (ABT), clotrimazole (CLOT), and 2,5-pyridinedicarboxylic acid (PCA), details of the metabolic pathway around tabersonine in hairy roots of *C. roseus* were elucidated. ABT specifically inhibited the formation of hörhammericine, while CLOT inhibited the accumulation of lochnericine. Using jasmonic acid in combination with the inhibitors suggests an inducible P-450 enzyme responsible for the formation of hörhammericine. The inhibitor study also revealed that both lochnericine and hörhammericine are 'turned over' in hairy root cultures.

Keywords: *Catharanthus roseus* L. (G. Don); Apocynaceae; Rosy periwinkle; Hairy roots; P-450 inhibitors; Tabersonine; Hörhammericine; Lochnericine

1. Introduction

Plant cell and tissue cultures of *Catharanthus roseus* have been studied extensively for the production of the anti-cancer compounds, vincristine and vinblastine (see Fig. 1) (Meijer, Verpoorte, & Hoge, 1993). These dimeric compounds are derived from the coupling of the monomers vindoline and catharanthine. Catharanthine is routinely detected in cell and tissue cultures (Kurz, Chatson, Constabel, Kutney, Choi, Kolodziejczyk, Sleigh, Stuart, & Worth, 1981; Drapeau, Blanch, & Wilke, 1987; Vazquez-Flota, Moreno-Valenzuela, Miranda-Ham, Voello-Coello, Loyola-Vargas, 1994), but the accumulation of stable and significant levels of vindoline has been elusive. Shoot (Krueger, Carew, Lui, & Staba, 1982; Endo, Goodbody, & Misawa, 1987; Constabel, Prairie, Kurz, & Kutney, 1982) and multiple shoot cultures (Hirata, Yamanaka, Kurano, Miyamoto, & Miura, 1987) are capable of producing vindoline and catharanthine, and vinblastine has been detected in multiple shoot cultures (Miura, Hirata, Kurano, Miyamoto, & Uchida, 1988). However, the growth rate of shoot cultures compared to the faster growing hairy root and cell suspension cultures prohibits their use in a bioprocess. Recently, stable production of vindoline was observed in cell cultures transformed with virulent *Agrobacterium* strains; although in relatively low amounts compared to the field grown plant (O'Keefe, Mahady, Gills, Beecher, & Schilling, 1997).

In non-transformed cell suspension cultures and hairy roots, the lack of expression of the enzymes between tabersonine and vindoline has generally been accepted to be the reason for the absence of vindoline (De Luca & Cutler, 1987). Potentially, a metabolically engineered system (Verpoorte, van der Heijden, & Memelink, 1996; Shanks, Bhadra, Morgan, Rijhwani, & Vani, 1998) in which the enzymes between tabersonine and vindoline are expressed may provide the solution to overcome this obstacle. In addition, optimal vindoline production would require minimizing flux that is diverted away from the enzymes of vindoline biosynthesis. Consequently, understanding of the flux

* Corresponding author. Tel.: 713-737-5877; Fax: 713-737-5877; E-mail: shanks@rice.edu

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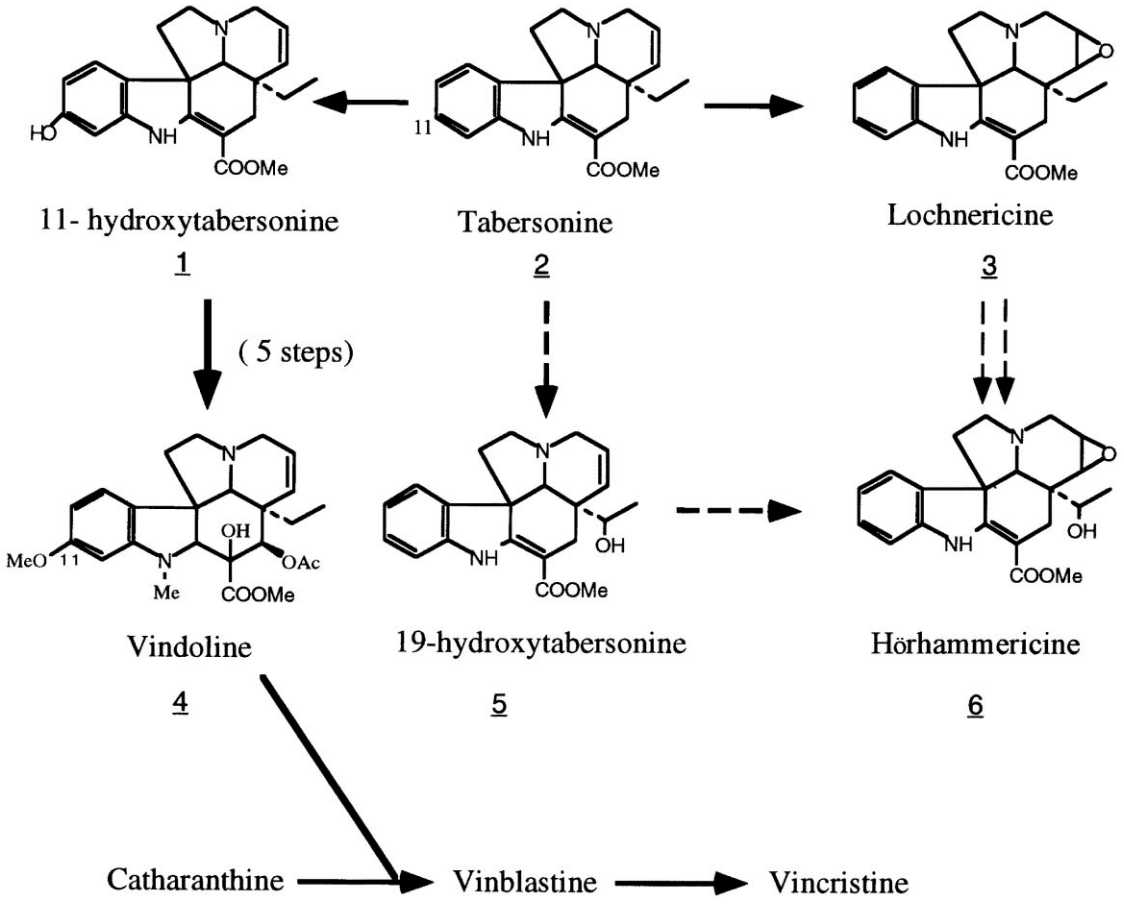

Fig. 1. Established and proposed biochemical conversions around tabersonine. The large solid arrow represents the final five steps in vindoline biosynthesis. The leftward solid arrow represents the P-450 dependent monooxygenase characterized in whole *C. roseus* plants (St.-Pierre & De Luca, 1995). The solid rightward arrow represents experimental evidence from Scott et al., 1973. The single slashed arrows are hypothesized pathways by Kutney et al., 1980. The double arrow represents another possible conversion to hörhammericine proposed in this paper.

through the pathways surrounding tabersonine is important in a metabolic engineering strategy. In cell and tissue cultures of *C. roseus*, there have been numerous reports of compounds, other than vindoline, which are likely derived from tabersonine (Scott, Reichardt, Slaytor, & Sweeny, 1973; Kurz, Chatson, Constabel, Kutney, Choi, Kolodziejczyk, Sleigh, Stuart, & Worth, 1980; Stöckigt & Soll, 1980). Previous feeding studies of tabersonine to *C. roseus* cell suspension cultures resulted in the accumulation of lochnericine, with further conversion to lochnerinine (Furuya, Sakamoto, Iida, Asada, Yoshikawa, Sakai, & Aimi, 1992). Similarly, in tabersonine feeding to seedlings, the major products detected were lochnericine and vindoline (Scott et al., 1973). In the hairy root line LBE 6–1, lochnericine and hörhammericine have been quantified in high amounts relative to tabersonine (Shanks et al., 1998). The transient accumulation of lochnericine and hörhammericine in hairy root cultures was previously examined from 18 to 70 days (Shanks et al., 1998).

The exact pathway and enzymes that convert tabersonine to lochnericine and hörhammericine are presently unknown. The formation of 11-hydroxytabersonine is the first step in vindoline biosynthesis from tabersonine; the enzyme responsible for this conversion has been characterized as a cytochrome P-450 monooxygenase (St.-Pierre & De Luca, 1995). Similarly, lochnericine and hörhammericine also differ from tabersonine by the incorporation of oxygen as shown in Fig. 1. Therefore, to investigate the epoxidation and hydroxylation of tabersonine, a dioxygenase inhibitor, 2,5-pyridinedicarboxylic acid (PCA) (Dowell & Hadley, 1992) and two P-450 dependent monooxygenase inhibitors, 1-aminobenzotriazole (ABT) (Reichart, Simon, Durst, Mathews, & Ortiz de Montellano, 1982) and clotrimazole (CLOT) were selected. Knowledge gained from inhibitor studies about the enzyme types involved in the reactions lead-

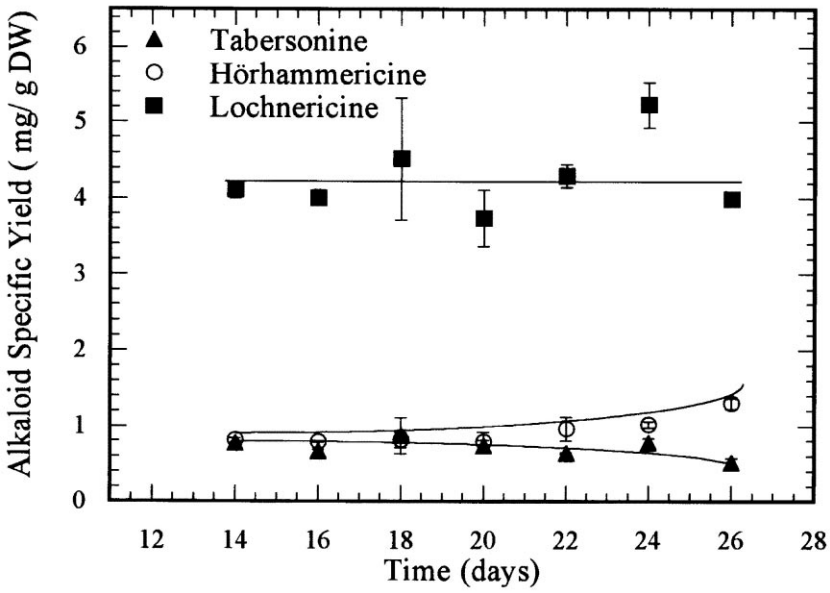

Fig. 2. Specific yields of (a) tabersonine (▲), hörhammericine (○), and (b) lochnericine (■) from hairy root cultures. Culture conditions: Gamborg's B5/2 media with 3% sucrose, 26°, shaken at 100 RPM in the dark. Symbols represent the mean of at least two data points, and error bars represent standard deviation.

ing to lochnericine and hörhammericine may provide clues on how to block these reactions. Jasmonic acid (JA) is known to increase flux to the alkaloids in *C. roseus* seedlings (Aerts, Gisi, De Carolis, De Luca, & Baumann, 1994; Aerts, Schäfer, Hesse, Baumann, & Susarenko, 1996) and hairy roots (Shanks et al., 1998). Thus, JA was used in combination with these inhibitors to examine the tabersonine branchpoint under conditions of enhanced flux and restricted ability to oxygenate tabersonine.

2. Results and discussion

Growth of the culture, as determined by increase in dry weight (DW) was remarkably linear ($r^2 = 0.99$) over the period between 14 to 26 days (data not shown). Transient profiles of the specific yields of tabersonine, lochnericine, and hörhammericine are shown in Fig. 2. Tabersonine specific yields which remain fairly constant, do decrease at 26 days. Hörhammericine specific yield mirrors that of tabersonine, with an increase at 26 days. Lochnericine accumulates to higher levels than tabersonine or hörhammericine for the entire period. Both lochnericine and hörhammericine total yields increased during the growth period of the roots (Fig. 3). Specifically, between 14 and 22 days the accumulation of all three alkaloids were linear.

The high specific yields of lochnericine and hörhammericine reported here are apparently not unique to hairy roots of *C. roseus*. Tabersonine, lochnericine, and hörhammericine were reported as the major alkaloids in both normal and hairy root cultures of *Catharanthus trichophyllus* (Davioud, Kan, Hamon, Tempe, & Henriphilippe, 1989). Tabersonine administration to cell suspension cultures resulted in its transformation to lochnericine as the major product (Furuya et al., 1992). Thus the diversion of flux at tabersonine away from the vindoline pathway commonly occurs in plant cell and tissue cultures, and may represent an impediment to the formation of maximal levels of vindoline in a metabolically engineered system.

2.1. Oxygenase inhibitors

Based upon the results of the transient data, the inhibitor experiments were conducted over the period of 14 to 21 days, which was a period of steady growth and alkaloid accumulation. This study was analyzed in terms of the effect of the inhibitor on specific yields of alkaloids, to reveal information about the enzyme types involved in the oxygenation of tabersonine. Further analysis of total alkaloid yield was used to examine turnover of lochnericine and hörhammericine.

As shown in Table 1, the addition of the P-450 dependent monooxygenase inhibitor, ABT, severely limited the formation of hörhammericine, increased the specific yield of tabersonine, and had no apparent effect upon the accumulation of lochnericine. In contrast to ABT, the addition of the dioxygenase inhibitor, PCA, had no significant effect upon the accumulation on any of these alkaloids, which indicates that an α-ketoglutarate dependent dioxygenase may not be involved in the biosynthesis of lochnericine

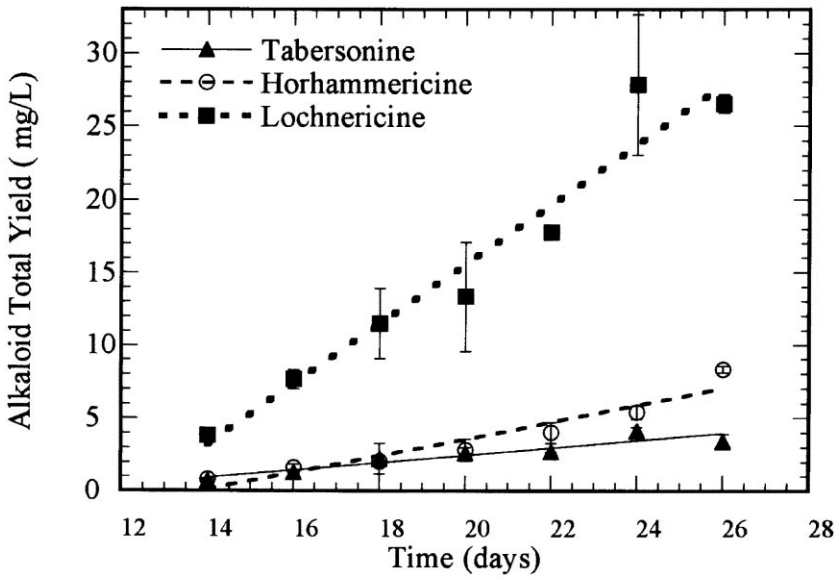

Fig. 3. Total yields of (a) tabersonine (▲), hörhammericine (○), and (b) lochnericine (■) from hairy root cultures. Culture conditions: Gamborg's B5/2 media with 3% sucrose, 26°, shaken at 100 RPM in the dark. Symbols represent the mean of at least two data points, and error bars represent standard deviation.

Table 1
Effect of addition of ABT and PCA upon specific alkaloid yields. ABT (0.5 μmol) and PCA (0.5 μmol) were added at day 10, and all cultures, including the control, were harvested at day 21. The values reported are the mean ± standard deviation. ($n = 3$)

Specific Alkaloid Yield (mg/g DW) Alkaloid	Control	Inhibitor ABT	Inhibitor PCA
Tabersonine	0.95 ± 0.16	2.89** ± 0.11	0.93 ± 0.12
Lochnericine	4.33 ± 0.19	4.39 ± 0.24	4.26 ± 0.52
Hörhammericine	1.27 ± 0.07	0.28** ± 0.02	1.24 ± 0.06

or hörhammericine. The lack of an effect could also be attributed to the inhibitor not reaching the compartment where the enzyme is present.

After observing that using PCA produced no significant effects, experiments were repeated using ABT and CLOT, another P-450 monooxygenase inhibitor. Table 2 shows the specific alkaloid yields before and after a 7 day treatment of a culture at 14 days with 1.25 μmol ABT, and 2.50 μmol CLOT. Significantly less hörhammericine was measured at 21 days in the ABT treated culture, again verifying that its synthesis is inhibited. In contrast to the effects of ABT, CLOT significantly inhibited the accumulation of lochnericine and hörhammericine. The combination of these results suggests that two different P-450 monooxygenase enzymes are involved in the formation of lochnericine and hörhammericine. Interestingly, tabersonine did not accumulate in the CLOT treated cultures as much as the ABT treatment. Perhaps this is partly due to the reduction in growth of the roots. However, as the

Table 2
Effect of addition of ABT and CLOT upon specific alkaloid yields. 1.25 μmol ABT and 2.50 μmol CLOT were added at day 14. The control cultures and cultures with inhibitor added were harvested at day 21. The values reported are the mean ± standard deviation ($n = 3$)

Specific Alkaloid Yield (mg/g DW) Alkaloid	Control	Inhibitor ABT	Inhibitor CLOT
Tabersonine	1.21 ± 0.04	2.49** ± 0.21	1.41* ± 0.07
Lochnericine	2.35 ± 0.17	2.41 ± 0.16	0.49** ± 0.07
Hörhammericine	0.49 ± 0.02	0.15** ± 0.01	0.25** ± 0.03

specific yield of lochnericine was significantly decreased, it appears that CLOT specifically inhibits the formation of lochnericine.

Each of these monooxygenase inhibitors was found to be detrimental to the growth of the root cultures at the levels used. Other P-450 inhibitors were found to have similar affects on cultured root growth (Sugimoto, Uchida, Inanaga, & Isogai, 1997). The lower total yields of tabersonine for the cultures treated with CLOT and the lower yields of lochnericine for the cultures treated with ABT at 21 days are apparently due to root growth inhibition (data not shown) as the specific yields were relatively unaffected (Table 2).

Inhibitor studies also have potential to readily observe the turnover of compounds for which formation is inhibited. Since the rate of accumulation is the rate of formation of a compound minus the rate of transformation and catabolism, transformation is more readily observable if the rate of synthesis can be reduced. Fig. 4 shows total yield of lochnericine from the same experiment reported in Table 2, but also includes the control at day 14. Total yields are reported, which allows comparisons of total alkaloid levels before and after the addition of inhibitor. The total yield of lochnericine accumulated at 14 days was significantly higher ($p < 0.01$) compared to the total yield present in the inhibited culture at 21 days, which indicates turnover of this compound. This turnover could either be in the form of transformation to hörhammericine and other products or catabolism. However, the total yield of hörhammericine did not significantly increase in cultures treated with either ABT or CLOT (data not shown). Furthermore, the presence of lochnerinine and hörhammerinine were not observed. Hörhammericine was also observed to be significantly transformed to other products or degraded during the period between 21 to 24 days in a culture treated with 5.0 μmol ABT at 21 days ($p < .01$) (Fig. 5). Thus the use of *in vivo* inhibitor studies complements other tools to investigate the important phenomenon of alkaloid turnover (Verpoorte, van der Heijden, Hoge, & ten Hoopen, 1994).

2.2. Elicitation and inhibition

Jasmonic acid (JA) is known to be an signal transducer which when applied results in increased accumulation of alkaloids in *C. roseus* (Aerts et al., 1994; Aerts et al., 1996). Specifically, in hairy roots, previous work has shown that the tabersonine branchpoint is highly responsive to treatment with JA (Shanks et al., 1998). Interestingly, JA application (0.25 mg/flask at 21 days) resulted in a significant increase in hörhammericine specific yield and a significant decrease in tabersonine specific yield after 100 hours. In the experiments reported in this paper, JA was added at 14 days at the same time as ABT and CLOT. Similar to the previous study, hörhammericine specific yield was dramatically increased, and tabersonine was significantly decreased after a 1 week treatment of JA ($p < 0.01$) (Table 3). Additionally, the accumulation of lochnericine was significantly decreased as a result of treatment with only JA ($p < 0.01$). The combination of JA and ABT had a similar outcome as the ABT treatments (Tables 1 and 2, and Table 3). There was a sig-

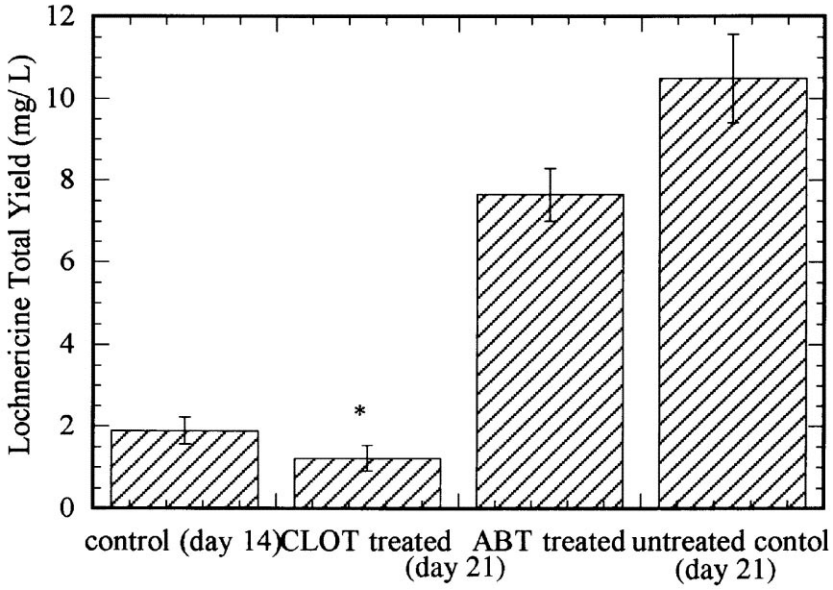

Fig. 4. The total yields of lochnericine for 2 week and 3 week control cultures, and for cultures treated with 2.50 μmol of CLOT or 1.25 μmol of ABT. * indicates that the total yield of lochnericine was significantly lower ($p < 0.01$) at 21 days in the CLOT treated culture, than the total yield at 14 days.

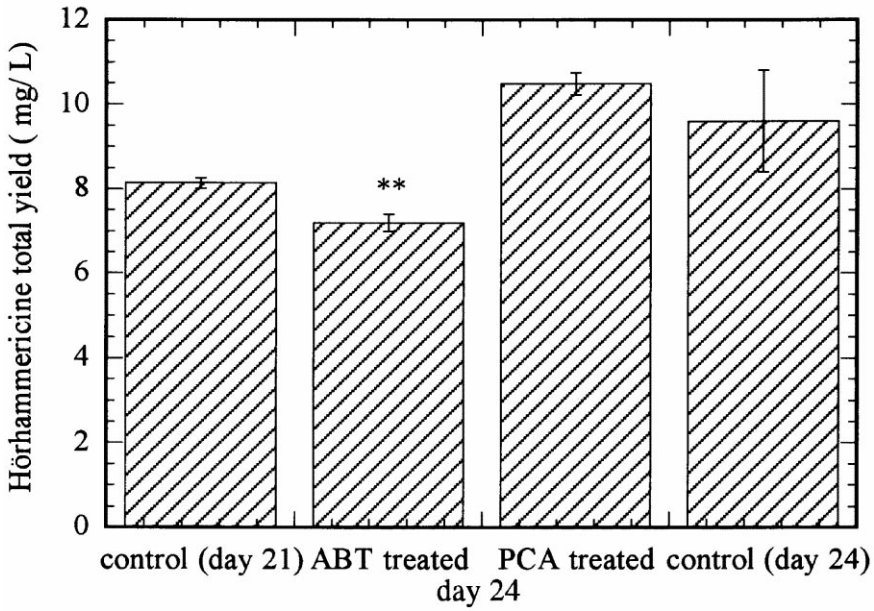

Fig. 5. The total yields of hörhammericine for 21 day and 24 day control cultures, and for cultures treated with 5.0 μmol of ABT or 5.0 μmol of PCA. ** indicates that the total yield of hörhammericine was significantly lower ($p < 0.01$) at 24 days in the ABT treated culture, than the total yield present at 21 days.

nificant reduction in hörhammericine specific yield, with significant increases in the specific yield of tabersonine ($p < 0.01$). In contrast to the previous ABT only treatments, a significant increase in lochnericine was observed compared to controls ($p < 0.01$). Notably the combination of CLOT and JA was unable to completely inhibit the increase in hörhammericine specific yield. Furthermore, the specific yield of tabersonine decreased, similar to the JA only treatment, rather than increase as expected for the CLOT only treatment. This result raises the possibility that in the elicited condition an inducible P-450 enzyme in the pathway to hörhammericine exists. Of particular relevance is a recent study that reports the cloning of a methyl jasmonate inducible cytochrome P-450 dependent monooxygenase involved in benzylisoquinoline alkaloid biosynthesis (Pauli & Kutchan, 1998).

The use of oxygenase inhibitors proved useful to study the tabersonine branchpoint. Similar studies have examined the biosynthetic pathways of taxol (Srinivasan, Ciddi, Bringi, & Shuler, 1996), brassinolides (Winter, Schneider, Strack, & Adam, 1997) and benzylisoquinoline alkaloids (Sugimoto et al., 1997). The pathway from tabersonine to lochnericine and hörhammericine is still not conclusively defined. From the results, ABT apparently strongly inhibits the formation of hörhammericine, but not the formation of lochnericine. Since ABT is a P-450 monooxygenase inhibitor, it likely prevents the hydroxylation of tabersonine. Furthermore, as only the specific yield of tabersonine was increased it suggests that hörhammericine may not absolutely require lochnericine as an intermediate. If hörhammericine was formed from lochnericine, higher levels of lochnericine would have been expected. The results from the inhibition with

Table 3
Effect of the combination of elicitation on hairy root cultures by jasmonic acid (JA), and addition of either ABT or CLOT upon specific alkaloid yields. At 2 weeks, 0.5 mg of JA was added to each treatment flask. At the same time 0.50 μmol ABT and 0.50 μmol CLOT were added to separate duplicate flasks, and the cultures were harvested at 21 days. Control cultures were harvested in triplicate. The values reported are the mean ± standard deviation.

Specific Alkaloid Yield (mg/g DW) Alkaloid	Control	JA	JA and ABT	JA and CLOT
Tabersonine	0.47 ± 0.05	0.22 ± 0.01	2.24 ± 0.39	0.29 ± 0.05
Lochnericine	3.28 ± 0.04	1.88 ± 0.11	6.99 ± 0.56	2.14 ± 0.13
Hörhammericine	0.48 ± 0.05	5.31 ± 0.10	0.71* ± 0.21	3.64 ± 0.18

*no significant difference between treatment and control at $p < 0.05$

CLOT are ambiguous with regard to the pathway. The suppression of both lochnericine and hörhammericine levels isn't indicative of either a route to hörhammericine through lochnericine or via a 19-hydroxytabersonine intermediate. Furthermore, the increase in hörhammericine yield in cultures treated with both JA and CLOT suggest the existence of a pathway inducible by jasmonic acid for the formation of hörhammericine. Therefore, the possibility of hörhammericine formation from either lochnericine or 19-hydroxytabersonine (Fig. 1) can not be ruled out.

The inhibitor ABT had been shown to be specific in reducing the activity of the P-450 type enzyme, cinnamic acid 4-hydroxylase (C4H) (Reichhart et al., 1982). As ABT is also an effective inhibitor of the formation of hörhammericine, C4H and the enzyme catalyzing this hydroxylation of tabersonine may have similarity. Interestingly, geraniol 10-hydroxylase (G10H), a cytochrome P-450 involved in the formation of the indole alkaloids of *C. roseus* was apparently not greatly inhibited by the ABT or CLOT treatment. Evidence for this comes from the combined specific yield of tabersonine, lochnericine, and hörhammericine was not reduced in the period from 10 to 21 days for the ABT treated cultures (Table 1). Furthermore the combined specific yields of ajmalicine and serpentine, two alkaloids derived from a pathway where G10H is the only known P-450 type enzyme involved, were suppressed only 30% by ABT treatment, and 16% by CLOT (data not shown). This may be due to the subcellular localization of G10H or the specificity of the inhibitor for differing monooxygenases.

In conclusion, the diversion of flux at the tabersonine branchpoint may be an impediment to the production of vindoline in transgenic cell or tissue cultures of *C. roseus*. The realization of significant vindoline production from cell or tissue cultures obviously requires the expression of the enzymes involved in vindoline biosynthesis. Additionally, it may be desirable to block the enzymes leading to lochnericine and perhaps hörhammericine. Whether or not the overexpression of the enzyme for the first step in vindoline biosynthesis from tabersonine would successfully compete for tabersonine is a critical future test. Further enzymological work is also required to clearly define the pathways leading to lochnericine and hörhammericine.

3. Experimental

3.1. Culture conditions

The hairy root line LBE 6-1 was used for all experiments (Bhadra & Shanks, 1997). The maintenance media consisted of a filter-sterilized 3% (w/v) sucrose solution, Gamborg's B5/2 mineral salts and vitamins. The initial pH of the solution was adjusted to 5.7. Cultures were initiated every 21 days by placing 5 root tips (35–40 mm) in 250 ml flasks containing 50 ml of media, which were shaken at 100 RPM, and kept at 26° in the dark.

3.2. Alkaloid analysis

The extraction and sample preparation procedures were simplified from those previously reported [30]. The crude MeOH extract was rotary evaporated to a volume of approximately 4 ml, and then passed through a 0.22 μm, 13 mm filter, of which 10 μl were injected on the HPLC column. The HPLC method was modified slightly to reduce total run time. The mobile phase consisted of a 32:32:36 mixture of MeOH: MeCN: 5 mM $(NH_4)_2PO_4$ buffer. An initial flow rate of 1 ml/min was maintained for 20 min, and linearly ramped for 10 min to 1.4 ml/min. The flow rate was returned to 1 ml/min over the next five minutes, where it was held for 5 minutes. Lochnericine and hörhammericine were identified by comparing their UV-vis, proton NMR spectra and MS fragmentation patterns against literature values (Kohl, Witte, & Höfle, 1981). As lochnericine and hörhammericine have the same chromophore as tabersonine, they were quantified at 329 nm using the tabersonine calibration curve. The tabersonine standard was a gift from Dr. Hamada (Okayama University, Japan).

3.3. Oxygenase inhibitor study

Culture conditions remained the same as described earlier. At 10 days in the culture cycle, filter sterilized inhibitors (ABT and PCA) were added to make a 10 μM solution. These cultures were harvested at 21 days. To independent cultures, 25 μM of ABT and 50 μM CLOT were added at 14 days, and these cultures were harvested at 21 days. ABT and CLOT were dissolved in a minimal amount of EtOH. All treatments were performed in triplicate, and an equal volume of filter sterilized water or EtOH was added to controls.

3.4. Statistical analysis

Differences between treated and control samples were analyzed by a students t-test using Microsoft Excel v.5.0 (Redmond, WA).

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