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BIOTRANSFORMATION OF TABERSONINE IN CELL SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS**

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Key Word Index—*Catharanthus roseus*; *Amsonia tabernaemontana*; Apocynanceae; indole alkaloids; vindoline; lochnericine; lochnerinine; epoxidation; cell suspension culture; biotransformation.

Abstract—To investigate the reactions involved in the biosynthesis of vindoline from tabersonine, the bioconversion products formed when the latter compound was fed to cell suspension cultures of *Catharanthus roseus* were isolated and characterized. Two biotransformation products of tabersonine were isolated and shown to be lochnericine, which is formed by epoxidation of tabersonine at positions 14, 15, and lochnerinine, the 11-methoxylation product of lochnericine. The bioconversion ratio of the main biotransformation product, lochnericine, reached a value of 80.6% within three days.

INTRODUCTION

Catharanthus roseus L. produces antimitotically active indole alkaloids, such as vincristine and vinblastine, which are widely used for cancer chemotherapy in humans. The dimeric alkaloids are synthesized from equimolar equivalents of the monomers vindoline and catharanthine in plant cells.

Tabersonine is a key precursor on the biosynthetic pathway leading to vindoline [1]. Young leaves of *C. roseus* plants are known to contain the enzymes which bioconvert the indole alkaloid, tabersonine, by two hydroxylations, an *O*-methylation, an *N*-methylation, an oxygenation and an *O*-acetylation step to vindoline [2–4]. Recently, the biosynthetic steps have been investigated at the enzymatic level and by *in vivo* experiments carried out with seedlings of *C. roseus* [5–8]. A number of research groups have attempted to obtain the production of the major alkaloids found in leaves of *C. roseus* by plant tissue culture techniques [9–12]. However, although the production of catharanthine has been demonstrated in cell suspension cultures, the biosynthesis of vindoline or the dimeric indole alkaloid derivatives has not been unequivocally obtained until recently [13–15]. It has been reported that the accumulation of vindoline was detected in cultured cells of *C. roseus* [16]. Also, the biosynthetic capacity of shoots regenerated from cell cultures to produce vindoline has been noted [17]. The pathway which leads to vindoline biosynthesis was expressed under light regulation [18]. By contrast, the ability of cell cultures to produce tabersonine has been demonstrated [19, 20]. We have investigated the ability of chloroplast-containing cell suspension cultures of *C. roseus* to form vindoline from tabersonine (**1**). Purification of the biotransformation products has been performed by HPLC, and identification of the products was carried out by means of ^1H and ^{13}C NMR and EI mass spectrometry. The biotransformation ratio, which is the ratio of the amount of the isolated product and the amount of administered **1**, was calculated by considering their M_rs.

RESULTS AND DISCUSSION

Induction of green coloured callus

Two to four weeks after culturing leaves and stems of *C. roseus* on DK medium (see Experimental) at 25° in the dark, creamy coloured and friable callus was induced. The callus was maintained on the same medium and subcultured under the same conditions at three-week intervals. After subculturing for several generations, the callus was transferred on to four different media, M-1, M-2, M-3 and M-4. By subculturing on the same medium over three generations, four differently greening calli were established, which were named M-1, M-2, M-3 and M-4. The green colour of the calli increased in the order M-4, M-2, M-1, and M-3. M-4, M-2 and M-1 were compact calli, while M-3 was a friable callus. M-4 was the fastest growing.

In a preliminary study, indole alkaloids, such as vindoline, catharanthine and their dimers, were not detected (TLC and HPLC) in the crude extracts of these four calli.

Identification of the biotransformation products

Tabersonine (**1**) hydrochloride (total 150 mg) was incubated for seven days with M-1 cell line which had been

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pre-cultured for two weeks. After harvest, the cells and medium were extracted and independently partitioned with organic solvents. Two principal peaks were detected on HPLC of the ethyl acetate extracts from the cells administered tabersonine (Fig. 1B). Traces of the two peaks and tabersonine were also observed in the control cells (Fig. 1A); however, the cell content of each of these compounds was very low (below *ca* 0.01% dry wt). Products **I** (R_t 20.2 min) and **II** (R_t 19.0 min) were isolated as crystals from these extracts by means of HPLC and by repeated recrystallizations.

Product **I** had the composition $C_{21}H_{25}N_2O_3$ (HRMS). In the ^1H NMR spectrum, the disappearance of olefinic proton signals which appeared at δ5.85 (H-14) and 5.23 (H-15) in tabersonine were observed. The presence of an epoxy ring was suggested on the basis of the signals at δ54.0 and 57.2 in the ^{13}C NMR spectrum of product **I**. The ^{13}C NMR spectral data of product **I** agree very closely with those of lochnericine (Table 1) [21]. Based on the physical and spectral data, product **I** was identified as lochnericine (**2**) [22–24].

The EI mass spectrum of product **II** showed a molecular ion peak at *m/z* 382 which was larger by 30 mass units than that of product **I**. In the ^1H NMR spectrum, the methoxyl protons appeared at δ3.77 (3H, *s*) and three aromatic protons were observed at δ 6.37 (1H, *dd*, $J=8$, 2Hz), 6.40 (1H, *d*, $J=2$Hz) and 7.01 (1H, *d*, $J=8$ Hz), indicating that the OMe substituent is attached to C-11. The ^{13}C NMR spectrum of product **II** was similar to that of product **I** except for the aromatic and the methoxyl carbon signals, suggesting that product **II** is the 11-methoxy derivative of product **I**, lochnerinine (**3**). Based on the physical and mass spectral data, product **II** was identified as lochnerinine (**3**) [22–24].

Consequently, the biotransformation pathway of **1** by cell suspension cultures of *C. roseus* is presumed to be as shown in Scheme 1.

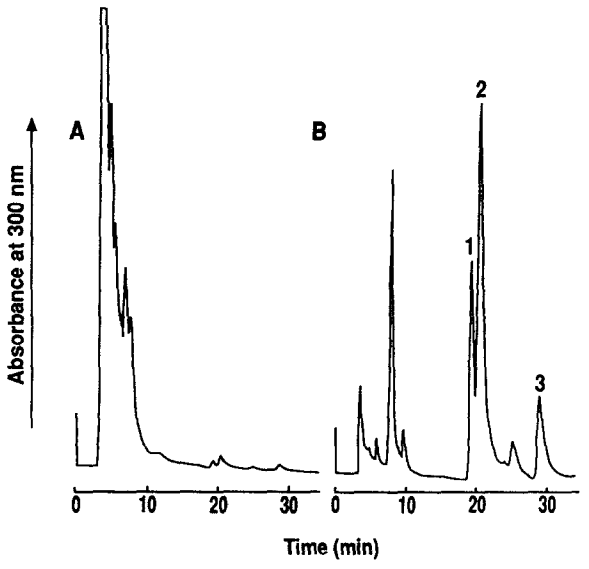


Fig. 1. HPLC analysis of alkaloid extract from cultured cells of *C. roseus* (A, control; B, after administration of tabersonine). The chromatographic conditions are described in the Experimental. Peak 3 (R_t 28.8 min) is the administered tabersonine.

Table 1. ^{13}C NMR chemical shifts of the biotransformation products **I** and **II** ($CDCl_3$, 100 MHz) and values from the literature

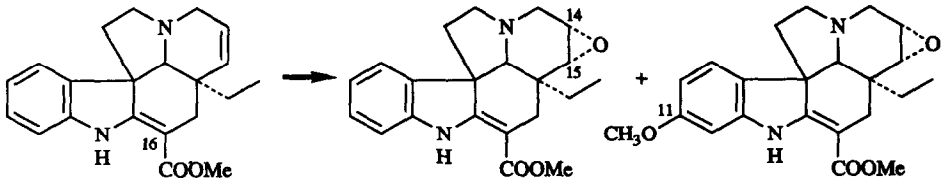
C	I	II	2	4
2	167.7	168.1	167.4	164.9
3	50.1	50.1	50.0	49.4
5	50.6	50.6	50.5	51.0
6	44.7	44.8	44.6	43.9
7	54.9	54.2	54.8	54.7
8	137.5	127.5	137.2	137.5
9	121.4	121.8	121.2	121.3
10	120.7	105.0	120.5	120.3
11	127.7	160.1	127.5	127.6
12	109.4	96.9	109.2	109.2
13	143.0	144.2	142.7	142.9
14	54.0	54.0	53.8	52.0
15	57.2	57.2	57.1	56.2
16	90.6	90.9	90.4	90.4
17	23.2	23.2	23.5	23.5
18	7.2	7.2	7.2	7.1
19	24.4	24.3	24.3	26.5
20	41.0	41.1	40.9	37.0
21	67.6	67.7	67.4	70.9
C=O	168.9	168.9	168.5	168.6
OMe	51.1	51.1	50.9	50.8
OMe		55.5		

*Data for **2** (lochnericine) and **4** (pachysiphine) are cited from ref. [21].

Compounds **2** and **3** have been found in many plants belonging to the Apocynaceae, such as the genera *Alstonia* [25], *Melodinus* [26] and *Tabernaemontana* [27] and also in cultured cells of *C. roseus* [28]. It is very interesting that Eilert *et al.* [29, 30] were able to induce accumulation of **2** in a cell suspension culture of *C. roseus* upon treatment of the cultures with homogenates of various fungi.

Cell growth and alkaloid production

The growth index and alkaloid production curves of a two-week-old M-1 cell line administered precursor **1** and then maintained under a light (12 hr)/dark (12 hr) regime are shown in Fig. 2. Since the growth of the two-week-old cultures was nearly at the stationary phase, the fresh weight of the suspended cells remained unchanged during the incubation period. Upon administration of **1** to the suspended cells, the accumulation *de novo* of the biotransformation product **I** continued up to day 3, and was proportional to the time of incubation. The biotransformation ratio at day 3 was 80.6% (in the cells). Compounds **2** and **3** were scarcely released into the medium and only 0.01–0.5% was detected in the ethyl acetate extract of the medium. After three days, the conversion of **1** to **2** gradually decreased. The production of **3**, which is formed by hydroxylation and *O*-methylation of **2**, was observed after six days. In leaves of *C. roseus*, **1** is converted first to 11-methoxytabersonine by hydroxylation and then *O*-methylation. However, in this experiment using suspension cultures, an epoxidation at posi-



Tabersonine (1) Lochnericine (2) Lochnerinine (3)

Scheme 1. Biotransformation of tabersonine in suspension culture of *C. roseus*.

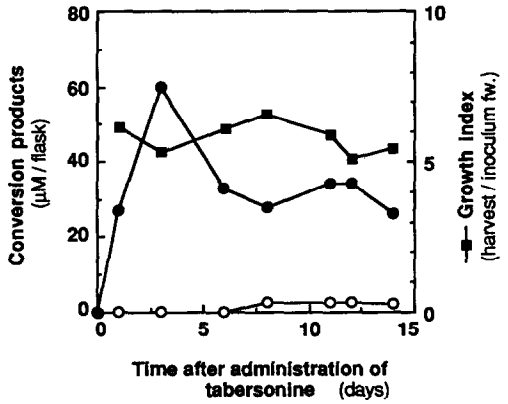


Fig. 2. Time course of cell growth and accumulation of the biotransformation products, lochnericine (●) and lochnerinine (○), in suspension cultured cells of *C. roseus*.

Table 2. Effect of different media on the biotransformation of tabersonine in suspension cultures of *C. roseus* after an incubation period of one week

	Biotransformation ratio (%)		
Medium*	Lochnericine	Lochnerinine	Total
M-1	61.9	7.3	69.2
M-2	43.1	12.0	55.1
M-3	0.7	0.9	1.6
M-4	1.3	n.d.†	1.3

*M-1; RT medium (1 mg l^{-1} NAA + 0.1 mg l^{-1} kinetin + 0.1% casamino acids) M-2; RT medium (1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin + 0.1% casamino acids) M-3; MS medium (1 mg l^{-1} NAA + 0.1 mg l^{-1} kinetin) M-4; MS medium (0.2 mg l^{-1} IAA + 2 mg l^{-1} 6-BA).

†n.d.; Not detected.

tion C-14 and C-15 of **1** preceded these two reactions by some considerable time.

Influence of medium on the biotransformation of tabersonine (**1**)

The effect of the medium on the biotransformation of **1** by suspension cultures under illumination, 12 hr light, is shown in Table 2. There were clear differences in the biotransformation ratio. The higher value was obtained on RT medium (see Experimental) containing 0.1% Casamino acids. The total amounts of all of the biotransformation products after one week incubation were 40- to 50-fold higher than those on MS medium lacking Casamino acids. Although the M-4 callus had the deepest green colour, the biotransformation ratio was the lowest of the calli. These results suggested that the bioconversion reaction of **1** to **2** and **3** was independent of the chloroplast content in the cells, and not related to cell growth. The response of the biotransformation reaction seemed to be dependent on the nutrients present in the culture medium. The effect of nutrient-limitation in the culture medium has been previously observed during biotransformation studies with cell cultures of other plants [31]. In this experiment, we could not detect the accumulation of vindoline produced by the biotransformation of **1**.

EXPERIMENTAL

General. Mp uncorr; ^1H (400 MHz) and ^{13}C NMR (100 MHz): $CDCl_3$; EIMS: 20eV; HPLC (biotransformation products and the other indole alkaloids): ODS (300 × 10 mm) column coupled to a UV detector and a differential refractometer. Compounds were detected on developed chromatograms by fluorescence quenching (plainly 254 or 365 nm) or visualized with Ce (IV) $(NH_4)_2SO_4$ (CAS) (1% in 85% H_3PO_4); R_f and colours (CAS spray on TLC) of products are given.

Plant material and cell culture conditions. The cultured cells used in this work were initiated in May 1981. Young leaves and stems of *C. roseus* (L.) G. Don were surface-sterilized for 10 sec with 70% EtOH, then for 10 min with 10% (w/v) bleaching powder. After rinsing with sterile dist. H_2O, the explants were placed on DK medium, i.e. Murashige and Skoog's (MS) medium [32] containing 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 3% sucrose and 0.9% agar (pH 5.8) at 25° in the dark. After the isolated callus had been subcultured on the same medium for several generations, the callus was transferred onto 4 media with different growth regulators and/or medium additives as follows. M-1 medium, Revised Tobacco (RT) medium [33] containing 1 mg l^{-1} NAA, 0.1 mg l^{-1} kinetin and 0.1% Casamino acids; M-2, RT medium (1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin and 0.1% Casamino acids); M-3, MS medium (1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin); M-4, MS medium (0.2 mg l^{-1} IAA and 2 mg l^{-1} 6-benzylaminopurine). The 4 calli were subcultured at 25° under illumination (12 hr light and 12 hr dark) at 2000–3000 lux (fluorescent lamp).

Preparation of tabersonine. Extraction and separation of tabersonine from *Amsonia tabernaemontana* Walt seeds was carried out according to a modified method of ref. [34]. The powdered seeds (3.12 kg) of *A. tabernaemontana* were extracted with MeOH (3.5 l × 4) at 70° for 8 hr and the MeOH extract concd *in vacuo*. The extract (427.6 g) was dissolved in 1 l EtOAc and extracted with 300 ml 1N HCl (× 4). The aq. layer was washed with 200 ml

EtOAc and made basic (pH 8.0) with $NaHCO_3$ in an ice bath. The alkaline soln was extracted with 400 ml EtOAc (×4) and the EtOAc layer washed with 200 ml satd NaCl soln (×2). The EtOAc extract (19.3 g) was obtained by evapn of the solvent. Silica gel CC (700 g, Wako gel C-200) of the extract was carried out with $CHCl_3$–MeOH as solvent system. Altogether, 59 fractions (200 ml each) were collected and monitored by TLC on silica gel with $CHCl_3$ as developing solvent. Tabersonine was detected by spraying the plate with a CAS soln and heating for 2 min at 100°. Frs 10–30 (10.9 g) were further fractionated by silica gel CC (450 g, Wako gel C-200) using $CHCl_3$–MeOH as solvent and a total of 26 fractions (200 ml each) were collected and monitored as above. The tabersonine-containing fractions (frs 6–19) were collected and the organic solvent removed under red. pres. The residue was dissolved in 5 ml 1 N HCl and the aq. soln crystallized by dropwise addition of EtOH (40–60°). This procedure was repeated (yield 92%) and tabersonine·HCl (4.4 g) recrystallized as needles. The tabersonine was identified by direct comparison (HPLC, IR, UV, NMR, MS) with an authentic sample.

Administration of tabersonine and extraction procedure. Each callus (*ca* 7 g fr. wt) was transferred to 250 ml suspension culture medium (Table 2) and pre-cultured at 25° in light/dark condition in a rotary shaker (140 rpm) for 2 weeks. Tabersonine·HCl (25 mg) dissolved in 2 ml water was administered to each flask by filter sterilization (0.22 μm) and then the cell suspension cultures were incubated for different periods (1–14 days) under the same conditions. The suspended cells, which were incubated in the presence of tabersonine for different periods, were sepd from the media by filtration through a nylon cloth and lyophilized.

The pulverized cells were extracted with MeOH under reflux and the MeOH soln was concd under red. pres. The residue was dissolved in 1 N HCl and washed twice with EtOAc to remove the lipophilic components. The aq. layer was made alkaline with $NaHCO_3$ and further extracted with EtOAc to give a crude extract. The separated medium was made basic with NH_4OH and was extracted with EtOAc.

Detection of biotransformation products. Quantitative analysis of biotransformation products was performed by HPLC. The dried EtOAc extracts from cells and medium were dissolved in MeOH and chromatography was performed using an ODS (300 × 10 mm) column. The mobile phase was a MeCN–0.05 M NH_4OAc buffer (29:21 pH 4.0) at a flow rate of 4 ml min^{-1}. The products were detected by their UV absorption at 300 nm and were separated into 2 peaks (P1 and P2).

Isolation of lochnericine (2). After purification of P2 by HPLC (R_t 20.2 min), product I was recrystallized from MeOH to give prisms (6.1 mg), mp 193–195°, $[\alpha]_D^{20}$ – 505.9° (EtOH; *c* 0.10), IR ν_{max}^{KBr} cm^{-1}: 3375, 2850–2750, 1670, 1610; ^1H NMR (400 MHz, $CDCl_3$); δ 0.74 (3H, *t*, *J* = 7.5 Hz, H_3-18), 3.79 (3H, *s*, CO_2Me), 6.80–7.15 (4H, *m*, arom. H), 8.93 (1H, *br s*, NH); ^{13}C NMR: see Table 1; EIMS *m/z* (rel. int.): 352 $[M]^+$ (64), 214 $[C_{13}H_{12}NO_2]^+$ (35), 138 $[C_8H_{14}NO]^+$ (100).

Isolation of lochnerinine (3). Product II was isolated from P1 (HPLC R_t 19.0 min) and was recrystallized from MeOH to give prisms (4.6 mg), mp 167–169°, $[\alpha]_D^{20}$ –86.4° (EtOH; *c* 0.22), IR ν_{max}^{KBr} cm^{-1}: 3375, 2850–2750, 1675, 1615; ^1H NMR (400 MHz, $CDCl_3$); δ 0.74 (3H, *t*, *J* = 7.5 Hz, H_3-18), 3.77 (3H, *s*, OMe), 3.79 (3H, *s*, CO_2Me), 6.37 (1H, *dd*, *J* = 8, 2 Hz, H-10), 6.40 (1H, *d*, *J* = 2 Hz, H-12), 7.01 (1H, *d*, *J* = 8 Hz, H-9), 8.89 (1H, *br s*, NH); ^{13}C NMR: see Table 1; EIMS *m/z* (rel. int.): 382 $[M]^+$ (89.2), 244 $[C_{14}H_{14}NO_3]^+$ (50.5), 138 $[C_8H_{14}NO]^+$ (100).

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