# 119. Alkaloid Production in *Catharanthus roseus* Cell Cultures. XI[1]). Biotransformation of 3′,4′-Anhydrovinblastine to Other Bisindole Alkaloids

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(30.III.82)

*Summary*

Biotransformation studies of 3′,4′-anhydrovinblastine (**1**) in *Catharanthus roseus* cell suspension cultures are reported. A detailed study of one cell line, coded at '916', has revealed that **1** is transformed to the natural bisindole alkaloids leurosine (**2**) and catharine (**3**) in approximately 25–30 and 9–16% yield, respectively. In 24–48 h, about 40% of **1** is utilized by the cells. Conditions for biotransformation of **1** have not been optimized so the above yields quoted are not final.

Previous publications from these laboratories [2–9] have demonstrated the biosynthetic capabilities of different tissue culture cell lines from *Catharanthus roseus* to produce various types of alkaloids within the *Corynanthé*, *Aspidosperma* and *Iboga* families. It was shown that serially cultured callus and cell suspension cultures derived from highly uniform explants (anthers of buds identical in developmental stage) can produce a different spectrum of alkaloids, and with appropriate optimization of growth conditions these selected lines can provide significantly higher alkaloid yields than those normally found in the intact plant. Another area of potential interest for the purpose of increasing cell yield of target compounds, and indeed for biosynthetic studies as well, concerns the use of such cell lines for biotransformation of appropriate substrates introduced into the culture medium at different stages of culture growth. Studies involving the transformation of various functional groups within organic compounds by plant tissue cultures have been reported [10], but compared to the well developed area of microbial transformation, much research is still required with such cultures. We would like to present our results which demonstrate the first observations with a *C. roseus* cell line and the synthetic bisindole intermediate, 3′,4′-anhydrovinblastine (**1**) [11].

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0018-019X/82/4/1271-08$01.00/0 

The 3′,4′-anhydrovinblastine (**1**), a bisindole compound readily prepared in the laboratory by coupling of the alkaloids catharanthine (**7**) and vindoline (**6**) [12], was selected for this study since its chemistry according to our previous investigations was well-understood [13]. Furthermore, its relationship to the clinically important anti-tumor drugs vinblastine (**4**), vincristine (**5**) and the natural bisindole alkaloids leurosine (**2**) and catharine (**3**) provided another stimulus for this study.

Several serially cultured cell lines have been propagated for the preliminary screening to determine their capability of biotransforming **1** into desirable products. Only four experiments with each line were necessary to ascertain whether productive biotransformation was occurring. For each line, these experiments were: *a)* two control experiments (24 and 72 h) involving only cells to determine whether alkaloids isolated were being produced by the cells or were metabolites of the precursor, *b)* cell line + **1** (3–5 mg), harvested 24 h after addition of substrate, and *c)* cell line + **1** (3–5 mg), harvested 72 h after addition of substrate. The results of these experiments with a number of established cell lines are given in *Table 1.*

Preliminary screening of these cell lines demonstrated that several lines were metabolizing 3′,4′-anhydrovinblastine (**1**). However, the determination of metabolic products was complicated since the cell lines were producing their own alkaloids, and these possessed retention times on TLC. plates which were very similar to the desired metabolic products. Therefore, for our further studies, we chose a line coded as '916'. This *C. roseus* cell line was somewhat unique: it exhibited satisfactory growth characteristics etc., but did *not* produce any of the alkaloids normally found

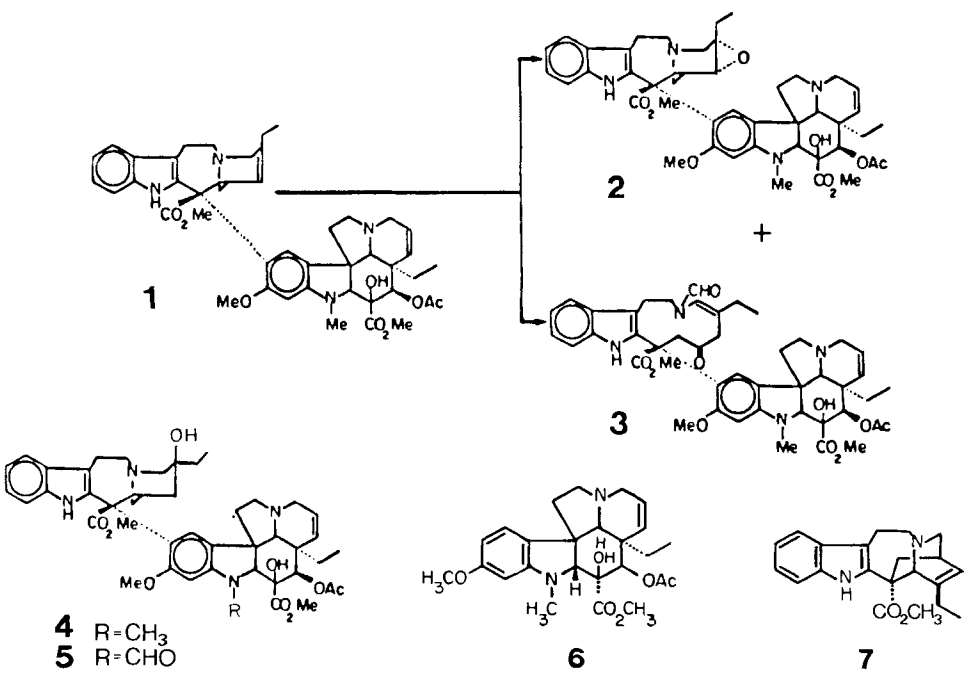

Table 1. *Small-scale biotransformation studies of 3′,4′-anhydrovinblastine* (**1**) *in* C. roseus *suspension cell cultures* (shake flasks)

| Cell line | Sample[a])[b]) | Incubation time [h] | Weight of freeze-dried cells (sample) [g] | Weight of basic alkaloid fraction [mg] | % of **1** in basic alkaloid fraction |
|---|---|---|---|---|---|
| 953 [9] | Control | 24 | 11.6 | 6 | – |
| 953 | **1** | 24 | 11.1 | 4 | < 1 |
| 953 | Control | 72 | 9.5 | 28 | – |
| 953 | **1** | 72 | 11.1 | 9 | 0 |
| 943 [4] | Control | 24 | 10.0 | 9 | – |
| 943 | **1** | 24 | 11.4 | 17 | 0 |
| 943 | Control | 72 | 10.6 | 9 | – |
| 943 | **1** | 72 | 10.6 | 48 | < 1 |
| 340 YG [7] | Control | 24 | 10.4 | 8 | – |
| 340 YG | **1** | 24 | 10.9 | 25 | 0 |
| 340 YG | Control | 72 | 10.1 | 11 | – |
| 340 YG | **1** | 72 | 11.0 | 17 | 0 |
| 200 GW [2] | Control | 24 | 11.2 | 38 | – |
| 200 GW | **1** | 24 | 10.6 | 43.8 | < 1 |
| 200 GW | Control | 72 | 11.2 | 5.4 | – |
| 200 GW | **1** | 72 | 10.3 | 13 | 0 |
| Growth medium | + **1** | 24 | 12.9 | 4.5 | 0 |
| Growth medium | + **1** | 72 | 13.6 | 2.3 | 0 |

[a]) Control means normal cell propagation without **1**.
[b]) In all experiments, **1** was added as hydrogensulfate salt.

in the other lines which we have investigated. A discussion about the propagation of this line will be published elsewhere.

In the initial study with the 916 cell line, 3–5 mg of 3′,4′-anhydrovinblastine (**1**) was incubated with the cells in shake flasks for 2, 6, 12, 18, 24, 48 and 72 h. The results of this study are summarized in *Table 2*. In the sample incubated for 2 h, mainly **1** was found; samples incubated for 6–72 h contained a new, less polar compound. However, the highest concentration of this new product was observed

Table 2. *Small-scale biotransformation studies of 3′,4′-anhydrovinblastine* (**1**) *in* C. roseus *suspension cell cultures* (shake flasks), *cell line 916*

| Incubation time [h] | Weight of freeze-dried cells [g] | Weight of basic alkaloid fraction [mg] | Percent of **1** in basic alkaloid fraction (by HPLC. and TLC.) |
|---|---|---|---|
| 2 | 7.24 | 21 | 15 |
| 6 | 9.00 | 17 | 0 |
| 12 | 4.80 | 12 | 0 |
| 18 | 6.11 | 15 | 0 |
| 24 | 6.60 | 12 | 0 |
| 24 | 5.48 | 16 | 0 |
| 48 | 4.80 | 15 | 0 |
| 48 | 2.90 | 11 | 0 |
| 72 | 5.40 | 6 | 0 |
| 72 | 5.02 | 11 | 0 |

in 24- and 48-h-incubation samples. In 72-h-incubation samples the concentration of the new product was decreasing and degradation products appeared. From these samples the new compound was subsequently isolated by TLC. and HPLC. methods. The initially isolated amount of material was sufficient to determine the dimeric nature of this product (UV.: $\lambda$max at 310, 290 and 160 nm) and its molecular weight (*m/z* 822.3819), which corresponds to the formula $C_{46}H_{54}N_4O_{10}$.

It became clear that *C. roseus* cell suspension cultures incubated with **1** produced very complex alkaloid mixtures. Therefore, development of appropriate HPLC. methods would be useful in analyzing such mixtures, and considerable effort was expended in this direction. A brief discussion of our investigations in developing the required HPLC. separation technique is presented.

**Analysis of alkaloid mixtures by HPLC.** – Alkaloid extracts were analyzed by HPLC. using a reverse phase *C-18* column, a methanol/water (0.1% $Et_3N$ as modifier) solvent system, and the UV. detector operating at both 254 and 280 nm. Initially, the isocratic solvent system with methanol/water 7:3 containing 0.1% $Et_3N$ as modifier, at a flow rate of 4 ml/min, was used for monitoring alkaloid mixtures. This solvent system provided a quick analysis (25–30 min) of the standard dimeric alkaloids found in *C. roseus* plant, *e.g.* 3′,4′-anhydrovinblastine (**1**), vinblastine (**4**), vincristine (**5**) and leurosine (**2**). Standard retention times are given in *Table 3.*

In order to obtain a better resolution within the short retention time region of the chromatogram, some modifications have been made *(Table 4).*

Table 3. *HPLC. standard retention times, methanol/water 7:3 containing 0.1% $Et_3N$ as modifier, flow rate 4 ml/min,* Radial-Pak A C-18 *cartridge*

| Alkaloid | | Standard retention time [min] |
|---|---|---|
| Catharine | (**3**) | 3.21 |
| Leurosine | (**2**) | 7.66 |
| 3′,4′-Anhydrovinblastine[a]) | (**1**) | 25.73 |
| Vinblastine[a]) | (**4**) | 5.38 |
| Vincristine[a]) | (**5**) | 3.73 |

[a]) Injected as the hydrogensulfate salt.

Table 4. *Modified solvent system for use with* Radial-Pak A C-18 *cartridge*

| Time [min] | Flow rate [ml/min] | $H_2O$[a]) [%] | $CH_3OH$[a]) [%] | Gradient curve |
|---|---|---|---|---|
| Initial | 1.40 | 30 | 70 | – |
| 20.00 | 4.00 | 30 | 70 | 11 |
| 40.00 | 1.40 | 30 | 70 | 11 |

[a]) Contains 0.1% $Et_3N$ as modifier.

With this new solvent program, adequate separation of most of the components could be accomplished at a longer run time (*ca.* 40 min.). Results are shown in *Table 5.*

**Studies with the 916 cell line (large scale).** – In our preliminary experiments, the best results were obtained after incubating 3′,4′-anhydrovinblastine (**1**) with the 916 cell line in shake flasks for 24 and 48 h. Therefore, for large-scale experiments with

Table 5. *HPLC. standard retention times, modified methanol/water solvent system (s.* Tab. 4*),* Radial-Pak A C-18 *cartridge*

| Alkaloid | | Standard retention time [min] |
|---|---|---|
| Catharine | (**3**) | 7.35 |
| Vincristine[a]) | (**5**) | 8.21 |
| Vinblastine[a]) | (**4**) | 11.55 |
| 3′,4′-Anhydrovinblastine[a]) | (**1**) | 36.12 |
| Leurosine | (**2**) | 17.00 |
| Vindoline | (**6**) | 7.34 |
| Catharanthine | (**7**) | 12.86 |

[a]) Injected as the hydrogensulfate salt.

the 916 cell line of *C. roseus*, cell suspension cultures (5.5 l) in a *Microferm* bioreactor and 24 and 48 h time periods were chosen.

In the initial large-scale experiments, the freeze-dried sample containing cells and remaining medium constituents was extracted with methanol and partitioned between neutral and basic fractions. The basic alkaloid fraction was subjected to semi-preparative HPLC. separation employing silica gel in 30 cm stainless steel columns (see *Exper. Part*) to provide two major and two minor components. Subsequent comparison of the spectral and chromatographic characteristics of the major components with authentic samples of leurosine (**2**) and catharine (**3**) established their identity. Based on recovered **1**, the yields of **2** and **3** in this biotransformation study were 31% and 9%, respectively. The other minor components, due to the small amounts available, could not be characterized.

It should be noted that appropriate blank experiments, conducted simultaneously with **1** in the growth medium *but* in the absence of cells, provided a very low yield of leurosine (4%) and traces of catharine (< 1%), thereby establishing that **2** and **3** are true biotransformation products and not artefacts formed in the medium.

It was found preferable, in subsequent studies, to separate the growth medium from the cells *prior* to isolation of the products from each component. The results thus obtained would also provide a relative comparison of compounds found in the medium *versus* those residing within the cells. Therefore, 3′,4′-anhydrovinblastine (**1**; 300 mg, added as the hydrogensulfate salt) was incubated with *C. roseus* cell suspension culture (cell line 916, 5.5 l) in a *Microferm* bioreactor for 48 h; then, cell material and culture medium were separated by filtration and freeze-dried separately. Freeze-dried samples were extracted according to the standard extraction procedure (s. *Exper. Part*) to give four extracts: the neutral cell material extract, the basic cell material extract, the neutral supernatant extract, and the basic supernatant extract. These fractions were analyzed by HPLC. and TLC. The initial crude extracts were purified by preparative HPLC. employing stainless steel columns and subsequently on smaller scale by TLC.

*The basic supernatant extract.* The crude basic supernatant extract was chromatographed over a *C-18* reverse phase packing. Alkaloids isolated were catharine (**3**) and leurosine (**2**). No 3′,4′-anhydrovinblastine (**1**) was detected. Several other

components isolated from this extract appeared to be not dimeric, according to MS. and UV. analyses.

*The basic cell material extract.* Reverse phase column chromatography gave the following dimeric alkaloids: catharine (**3**), leurosine (**2**) and 3′,4′-anhydrovinblastine (**1**). Other components isolated from this extract did not show dimeric alkaloid properties (UV. and MS. analyses).

*The neutral supernatant extract.* The standard reverse-phase column chromatography gave catharine (**3**), leurosine (**2**) and 3′,4′-anhydrovinblastine (**1**). Other isolated components were not dimeric alkaloids.

*The neutral cell material extract.* Preparative TLC. gave leurosine (**2**) and 3′,4′-anhydrovinblastine (**1**). Other components isolated from this extract were not dimeric.

The results of this experiment are summarized in *Tables 6* and *7*. Based on the amount of recovered substrate, the transformation of 3′,4′-anhydrovinblastine (**1**) to leurosine (**2**) and catharine (**3**) was 25.5 and 16.3%, respectively, or, approximately 42% of **1** had been utilized by the cells. It should be noted, however, that no attempts have yet been made to optimize the yields of specific products.

The above results also indicate that these high-molecular-weight alkaloids have passed through the cell walls since bisindole alkaloids were present in both the cell material and the culture medium.

Finally, the short period of time required for such biotransformations (24–48 h) is interesting, particularly when compared to plant cell culture production of alkaloids from nutrients present in the growth medium (usually several weeks). The

Table 6. *Biotransformation of 3′,4′-anhydrovinblastine* (**1**; 300 mg[a])) *in* C.roseus *suspension cell culture in* Microferm *bioreactor, cell line 916, 48 h*

| Sample | Weight of sample [g] | Weight of methanol extract [g] | Weight of neutral ethyl acetate extract [g] | Weight of basic ethyl acetate extract [g] |
|---|---|---|---|---|
| Cell material | 10.25 | 3.5 | 0.469 | 0.065 |
| Supernatant | *ca.* 140 | – | 0.143 | 0.187 |

[a]) Added as the hydrogensulfate salt.

Table 7. *Alkaloids isolated from biotransformation of 3′,4′-anhydrovinblastine* (**1**; 300 mg[a])) *in* C.roseus *suspension cell culture in* Microferm *bioreactor, cell line 916, 48 h*

| Alkaloid isolated | Weight of alkaloid isolated [mg] | | | | | % of the substrate added |
|---|---|---|---|---|---|---|
| | Basic supernatant extract | Basic cell material extract | Neutral supernatant extract | Neutral cell material extract | Total | |
| 3′,4′-Anhydrovinblastine (**1**) | – | 30.0 | 16.4 | 54.0 | 100.4 | 33.5 |
| Catharine (**3**) | 28.8 | 3.1 | 0.8 | – | 32.7 | 10.9 |
| Leurosine (**2**) | 17.0 | 8.2 | 17.2 | 8.6 | 51.0 | 17.0 |

[a]) Added as the hydrogensulfate salt.

inoculation of suspension cultures with biosynthetically 'advanced' precursors which reduce time periods for the production of target compounds may provide an important avenue for the commercial production of such pharmaceutically important agents. Further studies are underway.

## Experimental Part

*General.* Known alkaloids were identified by comparison of spectral data with that of authentic samples, by TLC. and by analytical high performance liquid chromatography (HPLC.). HPLC. was carried out using *Waters Associates* analytical system modified with: system controller (Model 720) data module, *Waters* intelligent sample processor *(Wisp)* (Model 710B), UV. absorbance detector (Model 440) working with dual channel detection at 254 and 280 nm, and radial compression module with *Radial-Pak A C-18* cartridge operating with reverse phase solvent systems.

**Standard extraction procedure.** – A typical extraction of freeze-dried cells was carried out as follows: freeze-dried cells (~ 10 g) were suspended in MeOH (400 ml) and extracted using an ultrasonic bath for 3–4 h. The suspension was filtered and the solid reextracted (twice 300 ml). The extracts were combined and evaporated *in vacuo* (temp. < 40°). The residue was suspended in 1N HCl (50 ml) and washed with AcOEt (4 times 50 ml). The combined AcOEt-extracts were washed with NaCl-solution, dried and concentrated to afford the crude neutral extract. The aq. solution was neutralized with $NaHCO_3$, the pH adjusted to 9.5–10.0 (1N NaOH) and extracted with AcOEt (4 times 50 ml). The combined extracts were washed with NaCl-solution, dried and concentrated to afford the crude basic alkaloid extract.

**Studies with the 916 cell line.** – *Experiment in which cells and medium were processed together.* In a *Microferm* Bioreactor 3′,4′-anhydrovinblastine (**1**; 500 mg, added as the hydrogensulfate salt) was incubated with the 916 line of *C. roseus* cell suspension culture (5.5 l) for 24 h. This provided a freeze-dried sample containing cells and medium constituents of 152.2 g. Extraction with methanol followed by acid-base partition of the crude methanol extract according to the standard extraction procedure (s. above) afforded a crude alkaloid fraction (520 mg). This was subjected to semi-prep. HPLC. purification over silica gel *(Waters Associates)* in a stainless steel column (30 × 2.5 cm) using $CH_2Cl_2/CH_3OH$ (gradient program) containig 0.1% $Et_3N$ as modifier, at a flow rate of 15 ml/min. A total of 40–50 ml fractions were collected and the column eluted with $CH_3OH$. The fractions were analyzed by HPLC. using *Waters Radial-Pak A C-18* cartridge (reverse phase) with $MeOH/H_2O$ 7:3 containing 0.1% $Et_3N$, at 1.4 ml/min and 4 ml/min, detection at both 254 and 280 nm. A dimeric alkaloid fraction (396 mg) was obtained. Trituration with cold methanol gave a residue consisting mainly of two components which were separated by prep. TLC. (pre-coated 20 × 20 cm plates (silica gel *60 $F_{254}$*), layer thickness 2 mm, developed 2 times with $CH_2Cl_2/CH_3OH$ 85:15 containing 0.01% $Et_3N$). Components were extracted from silica gel using an ultrasonic bath and $CH_2Cl_2/CH_3OH$ 3:1 containing 0.01% $Et_3N$. The two components were identified as *3′,4′-anhydrovinblastine* (**1**; 139 mg) and *leurosine* (**2**; 83 mg) by direct comparison of their spectral and physical properties with that of authentic samples. The mother liquor from the trituration was further purified by prep. TLC. as described above, to afford more **1** (25.6 mg) and **2** (23 mg) plus a less polar component, *catharine* (**3**; 29.7 mg). The structure of the latter was assigned on the basis of its spectral properties and confirmed by direct comparison with an authentic sample. Two other minor components exhibiting 'dimeric' alkaloid UV. absorption were also isolated. Complete elucidation of their structures has not been possible because of the limited amount of material available.

*Experiment in which the cells were separated from the growth medium.* In a *Microferm* bioreactor, 3′,4′-anhydrovinblastine (**1**; 300 mg, added as the hydrogensulfate salt), was incubated with the 916 cell line of *C. roseus* cell suspension culture (5.5 l) for 48 h. Cell material and culture medium were separated by filtration and freeze-dried separately giving 10.25 g of freeze-dried cells and *ca.* ~ 140 g of freeze-dried supernatant (growth medium). Freeze-dried cells were extracted according to the standard extraction procedure. This afforded the *neutral cell material extract* (469 mg) and the *basic cell material*

*extract* (65 mg). The freeze-dried supernatant sample was suspended in 1N HCl (100 ml) and washed with AcOEt (4 times 75 ml). The combined AcOEt-extracts were washed with NaCl-solution, dried and evaporated to afford the *neutral supernatant extract* (143 mg). The aqueous solution was neutralized with $NaHCO_3$, the pH adjusted to 9.5–10.0 (1N NaOH) and extracted with AcOEt (4 times 75 ml). The combined extracts were washed with NaCl-solution, dried and concentrated to afford the *basic supernatant extract* (187 mg). An intermediate-scale HPLC. system was employed for the separation of these extracts. A typical procedure is as follows. The crude AcOEt-extract was chromatographed over a *C-18* reverse phase packing in a stainless steel column (30×2.5 cm) with $CH_3OH/H_2O$ (solvent gradient program) containing as modifier 0.1% $Et_3N$, at a flow rate of 6 ml/min. A total of 40 fractions (50–75 ml) were collected for each separation, and the column was then eluted with $CH_3OH$ (300 ml). All fractions were weighed and analyzed by HPLC. using *Waters Radial-Pak A C-18* cartridge (reverse-phase) with $CH_3OH/H_2O$ 7:3 containing 0.1% $Et_3N$, at 1.4 ml/min and 4 ml/min, detection at both 254 and 280 nm. TLC. analyses were performed on analytical *KC18F* reverse-phase pre-coated plates (200 $\mu$ thickness) with $CH_3OH/H_2O$ 7:3, 8:2, and 9:1 as solvent. The HPLC. fractions with the same HPLC. profile were combined and further purified by prep. TLC. (20×20 cm silica gel plates, 1 mm layer thickness, solvent system $CH_2Cl_2/CH_3OH$ (solvent gradient)). Components were extracted from silica gel with $CH_2Cl_2/CH_3OH$ 3:1 containing 0.01% $Et_3N$. The chromatography of the *basic supernatant extract* yielded **3** (28.8 mg) and **2** (17.0 mg). The chromatography of the *basic cell material extract* gave **1** (30.0 mg), **3** (3.1 mg) and **2** (8.2 mg). The chromatography of the *neutral supernatant extract* gave **1** (16.4 mg), **3** (0.8 mg) and **2** (17.2 mg). Prep. TLC. of the *neutral cell material extract* afforded **1** (54.0 mg) and **2** (8.6 mg).

We are grateful for the skilled assistance of Miss *K. Busch*, Mrs. *P. Gaudet-La Prairie*, Mrs. *L. Leland* and Miss *S. Rambold*. The part of this research program performed at the University of British Columbia was supported by a *NRC* Research Contract under the Fermentation Technology Program (00-310-SX-8-3011) and by a *NSERC* grant.

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