198. Alkaloid Production in *Catharanthus roseus* Cell Cultures. IV[1]). Characterization of the 953 Cell Line

by **Wolfgang G.W. Kurz, Kenneth B. Chatson** and **Friedrich Constabel**

Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada S7N OW9

and **James P. Kutney, Lewis S.L. Choi, Pawel Kolodziejczyk, Stephen K. Sleigh, Kenneth L. Stuart** and **Brian R. Worth**

Department of Chemistry, University of British Columbia, Vancouver, B.C., Canada V6T 1Y6

(13.VI.80)

Summary

The 953 cell line, one of the cell lines from *Catharanthus roseus*, has been investigated in terms of its ability to produce indole alkaloids. Various parameters (mitotic index, pH, cell dry weight, growth period) were evaluated in the fermentation process and their relative importance for alkaloid production was determined. The alkaloids isolated and identified are ajmalicine, yohimbine, isositsirikine, vallesiachotamine, strictosidine lactam, lochnericine, hörhammericine, hörhammerinine, vindolinine, 19-epivindolinine, 19-acetoxy-11-methoxytabersonine, 19-hydroxy-11-methoxytabersonine and dimethyltryptamine.

In recent publications [1–3] we have reported the establishment of cell lines from *Catharanthus roseus* both as callus and cell suspension cultures. Many of these were capable of synthesizing complex indole alkaloids that occur late in the biosynthetic pathways. These results have been consistently reproducible over two years and the yields of specific alkaloids have been better than or comparable with those available from the plant.

The cultures were derived from highly uniform explants [1]. Variation among serial subcultures was readily observed. The resulting callus and suspension cultures represented cell lines uniquely characterized by their morphological and enzymatic differences. These differences allow both ready identification of, and stability checks for specific cell lines. Furthermore, the ability to produce predominantly one alkaloid or group of alkaloids from a single cell line is of great importance. Their immense potential as sources for compounds of industrial and pharmaceutical use and also as tools for biosynthetic studies can be readily understood.

Detailed studies of two such lines have been reported [2] [3]. The present work describes a particular cell line coded '953'.

[1]) For Part I–III: s. [1–3].

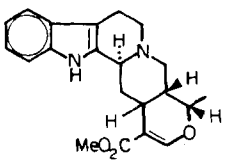
0018-019X/80/7/1891-06$01.00/0

Table 1. *Alkaloid Yields from Batches of 953 Line* C.roseus *Cell Cultures*

Sample	Culture method	Weight of freeze dried cells (g)	Weight of basic fraction (g)	% Alkaloid
1	Bioreactor (10 days)	90.5	0.168	0.185
2	Bioreactor (11 days)	110.0	0.178	0.16
3	Bioreactor (22 days)	26.9	0.058	0.21
4	Shake flask (14 days)	40.6	0.065	0.16
5	Shake flask (21 days)	49.66	0.182	0.37

Table 2. *Alkaloid Yields from 953 Line* C.roseus *Shake Flask Cultures*

Cultivation time	Weight of freeze dried cells (g)	Weight of basic fraction (g)	% Alkaloid
3 Weeks	65.9	0.15	0.23
4 Weeks	51	0.15	0.29
5 Weeks	87.6	0.24	0.28
6 Weeks	19.8	0.125	0.63
7 Weeks	19.7	0.1	0.51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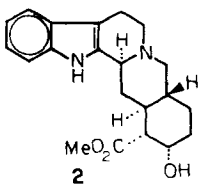
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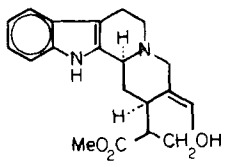
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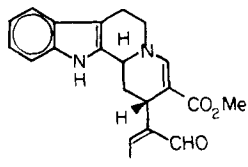
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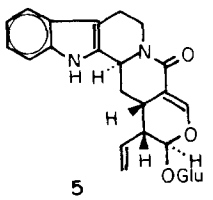
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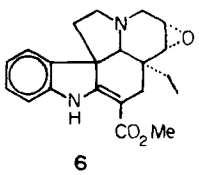
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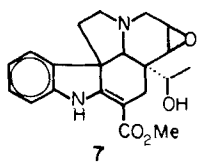
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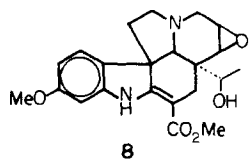
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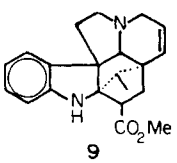
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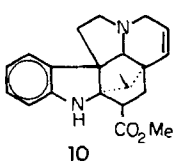
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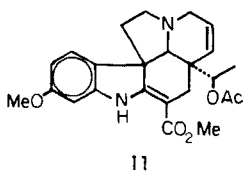
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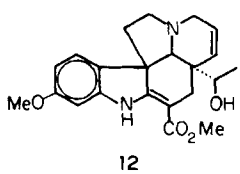
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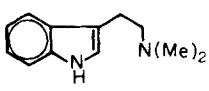
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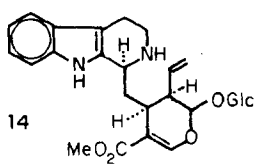
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The growth of the inoculum and its subsequent propagation in cell suspension was carried out as described earlier [2]. On harvesting the culture, the water was removed by freeze drying and the residue extracted. Separation of the alkaloids was achieved by the same chromatographic procedure used earlier [2]. The alkaloid yields from five such batchers are shown in *Table 1*. The crude alkaloid mixtures were fractionated by intermediate scale reverse phase high performance liquid chromatography (HPLC.). Final purification by analytical reverse phase HPLC. allowed the isolation of the following alkaloids, characterized by their physical and spectral data and by comparison with authentic materials: ajmalicine (**1**), yohimbine (**2**), isositsirikine (**3**), vallesiachotamine (**4**), strictosidine lactam (**5**), lochnericine (**6**), hörhammericine (**7**), hörhammerinine (**8**), vindolinine (**9**), 19-epi-vindolinine (**10**), 19-acetoxy-11-methoxytabersonine (**11**), 19-hydroxy-11-methoxy-tabersonine (**12**) and dimethyltryptamine (**13**).

Since general alkaloid formation was not observed during the initial periods of rapid cell growth, it was decided to examine whether the appearance, disappearance or build-up of particular components could be observed over different time periods. The results are given in *Tables 1* and *2* and *Figure 1*. These show that the percentage of alkaloid per gram of cell weight increases with time, with

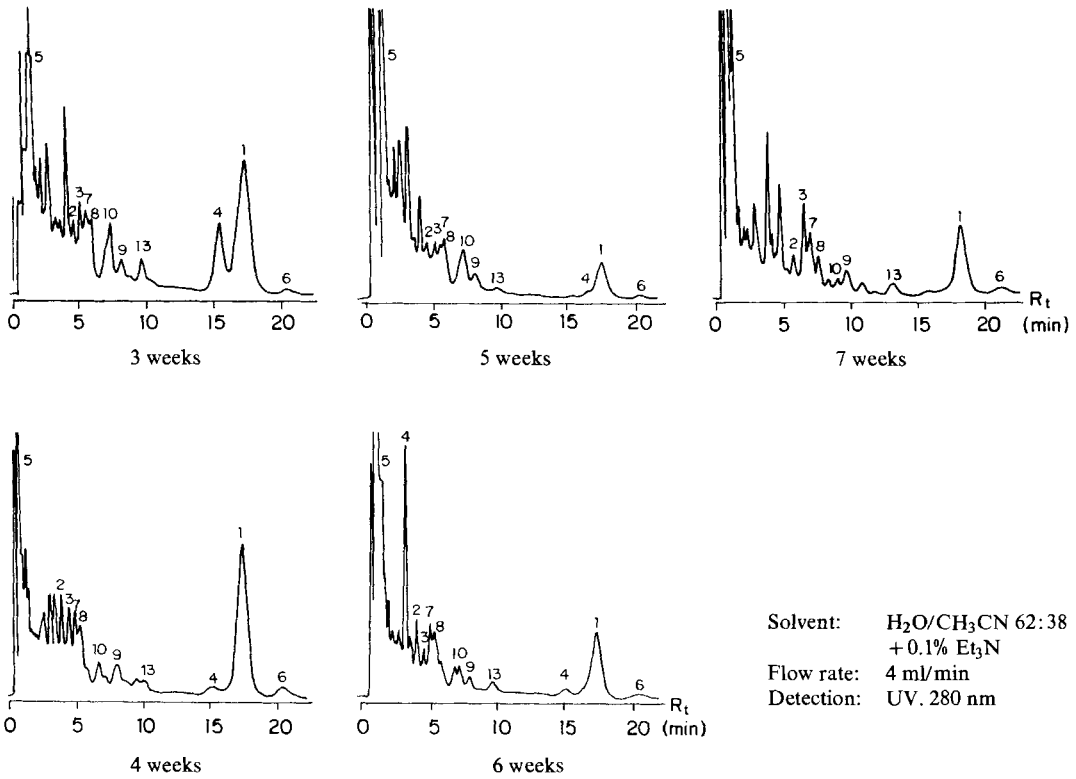

Fig. 1. *Reverse phase HPLC. of alkaloid mixtures obtained after different growth periods*

optimum production at 3–4 weeks. *Figure 2* supports this observation showing maximum cell dry weight occurring during the same period, coinciding with a zero value of the mitotic index. With respect to the earlier periods of culture growth *Figure 3* demonstrates a more rapid increase in the biosynthesis of ajmalicine (**1**) and yohimbine (**2**) (*Corynanthe* family) than observed for vindolinine (**9**) (*Aspidosperma* family). That is, the simple *Corynanthe* alkaloids ajmalicine (**1**) and yohimbine (**2**) reach maximum concentration at a much earlier period in *culture* growth than the biosynthetically more complex vindolinine (**9**). These are presumably derived from a common key intermediate, strictosidine (**14**), reflecting the differences in complexity of their biogenesis. *Figure 3* also shows that at *ca.* 25 days, the concentration of these alkaloids begin to equilibrate, coincident with the onset of cell autolysis *(Fig. 2). Figure 1* shows HPLC. traces of the later stages of growth

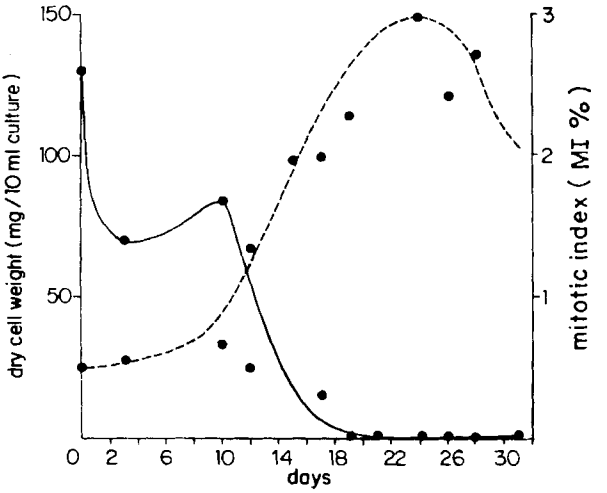

Fig. 2. *Dry cell weight (---) and mitotic index (—).*

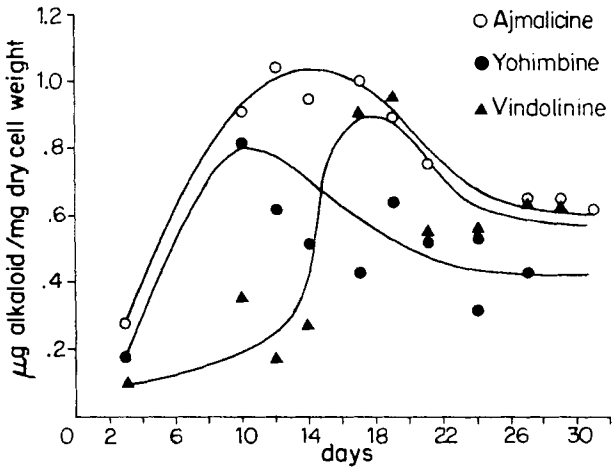

Fig. 3. *Content of Ajmalicine* (**1**), *Yohimbine* (**2**) *and Vindolinine* (**9**) *at the earlier periods of culture growth.*

period (3–7 weeks). Each sample contained ajmalicine (**1**) as the major component (*ca.* 15%). Furthermore, the analytical traces indicate that the other identifiable components of the mixture remained the same throughout this later period with only small changes in their relative concentrations.

These data uniquely characterize the 953 line and are being used to determine optimum production of specific alkaloids in other cell lines from *C. roseus.*

Experimental Part

General Remarks. All m.p. are uncorrected. Analytical HPLC. was carried out on a *Waters Associates* ALC 100 modified to incorporate a 440 UV. detector, and Data Module using dual channel detection at 254 and 280 nm. Separation was achieved using *Waters* Radial Compression Module fitted with a reverse phase pack, at a flow rate of 4 ml/min. Known alkaloids were identified by comparison of spectral data (MS., NMR., IR., UV.) with that of authentic samples, by mixed m.p. and by TLC. and co-injection on analytical HPLC.

Procedures for Culture Growth. The two procedures described below are typical conditions employed for the large scale propagation of the cultures in bioreactor and shake flasks. The details on initiation of callus and cell suspension cultures have been given earlier [1]. The cell line was subcultured on 1B5-medium, agitated on gyratory shakers (130 rpm) at 27° in continuous light (10 Wm^{-2}), and transferred weekly to fresh medium. This inoculum was then used to initiate all large scale productions of the cell line.

Growth in Bioreactor (Samples 1–3, Table 1*).* To 5 l of *Zenk*'s alkaloid production medium [4] in a 7.5-l-*Microferm* bioreactor, 500 ml of inoculum were added (ratio of medium to inoculum 10:1). The cultures were then grown under agitation at 200 rpm and aeration of 35 ml of air per l culture per min and at 27° for the chosen time period. Samples were withdrawn at various intervals and analyzed for mitotic index, cell dry weight and pH. The culture was then harvested and the water removed by freeze drying.

Growth in Shake Flasks (Samples 4 and 5, Table 1*).* The inoculum (100 ml) was transferred to a 500 ml Erlenmeyer flask containing 250 ml of *Zenk*'s alkaloid production medium and agitated on a gyratory shaker for the appropriate time period (3 weeks, 4 weeks *etc.*) and then harvested as above.

Standard Extraction Procedure. A typical extraction of freeze dried cells was carried out as follows: Freeze dried cells (90.5 g, sample 1, *Table 1*) were suspended in MeOH (500 ml) and extracted using an ultrasonic bath for 4 h. The suspension was filtered and the solid reextracted (2 × 500 ml). The extracts were combined and concentrated *in vacuo.* The residue was suspended in 1N HCl (200 ml) and washed with EtOAc (4 × 100 ml). The aqueous solution was neutralized ($NaHCO_3$), the pH adjusted to *ca.* 9.5 (1N NaOH) and the solution extracted with EtOAc (4 × 100 ml). The combined extracts were dried and concentrated to afford the crude alkaloids (168 mg). This mixture was further fractionated by trituration to give 140 mg of CH_2Cl_2 soluble material. The residue which was soluble in MeOH was not found in these cases to contain any alkaloids.

Alkaloid Isolation. A typical procedure is as follows: The crude CH_2Cl_2 soluble fraction (168 mg, sample 1, *Table 1*) was chromatographed over *Porasil B* (150 g, *Waters Associates*) in a stainless steel column (30 × 2.5 cm) with H_2O/CH_3CN 68:32 containing 0.1% Et_3N modifier, at a flow rate of 18 ml/min. A total of 30 50-ml fractions were collected and the column then eluted with CH_3CN (400 ml, fraction 31). The 31 fractions were analyzed by HPLC. (reverse phase packing, H_2O/CH_3CN 62:38 containing 0.1% Et_3N, at 4 ml/min, detection at both 254 and 280 nm). *Fractions 6* and *7* were combined and concentrated to give a yellow foam (14 mg). This afforded after purification by HPLC. (6 2-mg injections; radial compression pak B, H_2O/CH_3CN 65:35 with 0.1% Et_3N, at 4 ml/min, monitored at 254 nm) strictosidine lactam (**5**; 5 mg) identical with an authentic sample. Acetylation of this in the normal manner yielded its tetraacetate which was also found to be identical in all respects to an authentic sample. *Fraction 10* gave 4.5 mg of material which on purification by

HPLC. (3 1.5-mg injections, as before using H_2O/CH_3CN 40:60) gave a compound (3 mg) identical with an authentic sample of yohimbine (**2**). *Fraction 12* afforded 5 mg of material which on purification by HPLC. (3 1.7-mg injections) resulted in 4.5 mg of isositsirikine (**3**) identical with an authentic compound. *Fractions 13–18* when concentrated gave a pale cream froth (30 mg) which was purified by HPLC. (10 3-mg injections) to provide hörhammericine (**7**; 10 mg) identical with authentic material, and hörhammerinine (**8**; 12 mg) identical with an authentic sample. *Fractions 20–24* gave 18 mg of material which on purification by HPLC. (6 3-mg injections) provided 6 mg of 19-epivindolinine (**10**), and 9 mg of vindolinine (**9**) identical with respective authentic samples. *Fractions 25–28* afforded pure *N,N*-dimethyltryptamine (**13**; 3 mg) identical with an authentic sample. *Fraction 31* gave 72 mg of material, trituration with MeOH afforded ajmalicine (**1**; 30 mg) identical with authentic material. The remainder was concentrated and fractionated by HPLC. (7 6-mg injections) to give vallesiochotamine (**4**; 5 mg) identical with an authentic sample, 19-acetoxy-11-methoxytabersonine (**11**; 1 mg) identical with the sample previously isolated [2], 19-hydroxy-11-methoxytabersonine (**12**; 2 mg) identical with the sample previously isolated [2], and lochnericine (**6**; 3 mg) identical with an authentic sample.

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

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