ALKALOID PRODUCTION IN *CATHARANTHUS ROSEUS* CELL CULTURES. V. ALKALOIDS FROM THE 176G, 299Y, 340Y AND 951G CELL LINES[1]

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ABSTRACT.—Plant cell cultures, 176G, 299Y, 340Y and 951G, from *C. roseus* gave the following alkaloids: yohimbine (**5**), isositsirikine (**6**), horhammericine (**7**), horhammerinine (**8**), vindolinine (**9**), 19-epivindolinine (**10**), ajmalicine (**3**), lochnericine (**14**), vallesiachotamine (**4**), strictosidine (**12**), strictosidine lactam (**11**), lochnerinine (**15**) and N,N-dimethyl-tryptamine (**13**).

In recent publications (1–6) we have reported the production of a large number of indole alkaloids both in callus and suspension cultures derived from *Catharanthus roseus*. Among these cell cultures there have been cell lines that produce catharanthine (**1**, Iboga) and a variety of Aspidosperma and Corynanthé alkaloids. In a recent publication Stöckigt *et. al.* (7) have also reported the production of cultures that synthesize the more biosynthetically complex indole alkaloids. It was interesting to note that Stöckigt found, with a few minor differences, the same alkaloids that we had earlier isolated and *fully* characterized.

In our continued investigations for cell lines that produce catharanthine (**1**) and/or vindoline (**2**), we have been examining the differences found in alkaloid production from cell cultures that have been derived, by anther explants, from different *C. roseus* plants. By following this approach we hoped to propagate a cell line that could synthesize a single or, perhaps, two major components. A cell line that could produce predominantly a single chemo-therapeutic component would have considerable significance for the pharmaceutical industry. Here we describe some of the results so far obtained.

RESULTS AND DISCUSSION

The growing of each innoculum and its subsequent propagation in cell suspension was carried out as described earlier (2). When the culture was harvested, the water was removed by freeze drying and the residue was extracted. Separation of the alkaloids was achieved by the same chromatographic procedure used earlier (2).

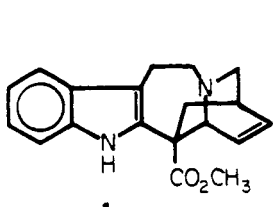
A culture derived from one plant and coded 176G was propagated; the alkaloids isolated are shown in table 1. The two major components are ajmalicine (**3**) (13.5% of total alkaloid content) and lochnericine (**14**) (8.5%), though there are significant amounts of yohimbine (**5**) (0.9%), isositsirikine (**6**) (0.75%), horhammericine (**7**) (1.85%), horhammerinine (**8**) (1.1%), vindolinine (**9**) (2.2%) and 19-epivindolinine (**10**) (2.0%).

A second cell line was derived from a plant coded 299Y. The alkaloid content is shown in table 2. In this case only two major alkaloids could be isolated: strictosidine (**12**) (0.35 g, 83% of total alkaloid content) and strictosidine lactam (**11**) (0.03 g, 6% of total alkaloid content). It is not clear at the moment whether

[1]NRCC #18655.

TABLE 1. Alkaloids isolated from the 176G cell line.

Alkaloid	Wt. (mg)	% of Total alkaloid content (270 mg)	% of Dry cell wt (62.1 gm)
Yohimbine (**5**)	2.5	0.9	0.004
Isositsirikine (**6**)	2.0	0.75	0.003
Horhammericine (**7**)	5.0	1.85	0.008
Horhammerinine (**8**)	3.0	1.1	0.005
Vindolinine (**9**)	6.0	2.2	0.009
19-Epivindolinine (**10**)	5.5	2.0	0.009
Ajmalicine (**3**)	25.0	13.5	0.04
Lochnericine (**14**)	16.0	8.5	0.026



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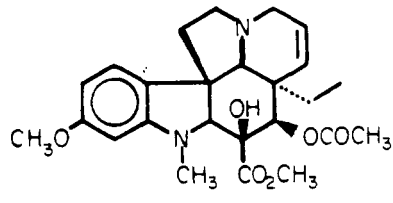

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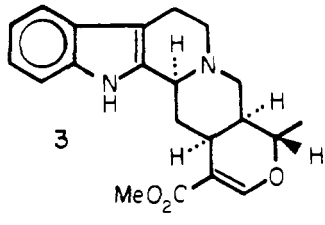

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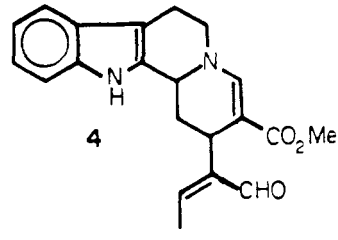

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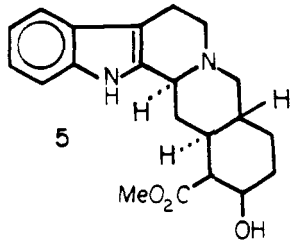

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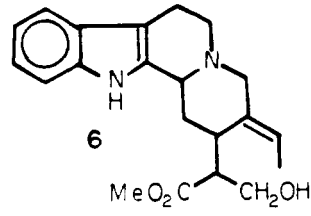

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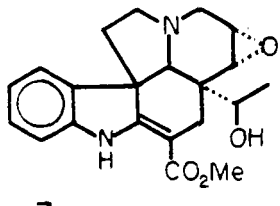

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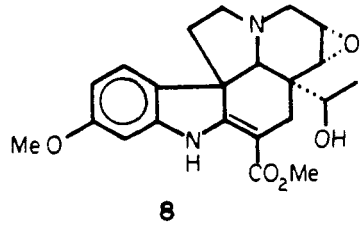

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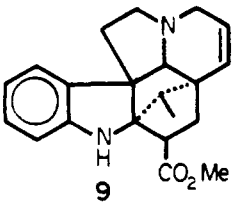

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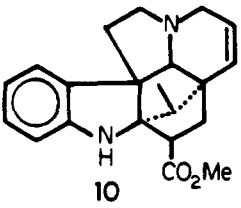

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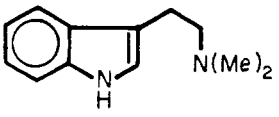

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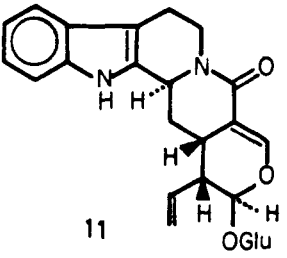

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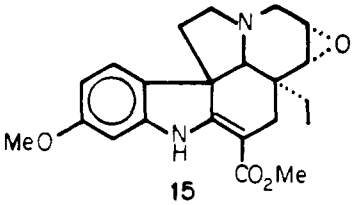

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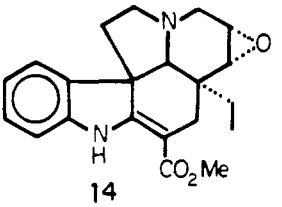

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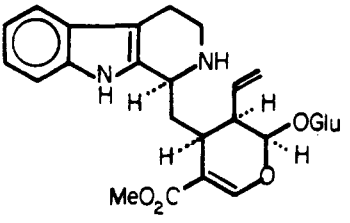

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(**11**) is present in the culture or is formed from (**12**) during the isolation procedure. Among the other components isolated were ajmalicine (**3**) (16 mg, 4.0%), vallesiachotamine (**4**) (17 mg, 4.2%), vindolinine (**9**) (.3 mg, 0.1%), horhammericine (**7**) (4 mg, 0.9%), horhammerinine (**8**) (2 mg, 0.5%), lochnericine (**14**) (2.5 mg, 0.6%) and lochnerinine (**15**) (2 mg, 0.5%). It is of interest that although strictosidine (**12**), the precursor of the indole alkaloids, was formed in abundance, the cell enzyme system did not seem to be able to transform it into more elaborate alkaloids.

TABLE 2. Alkaloids isolated from the 299Y cell line.

Alkaloid	Wt (mg)	% of Total alkaloid content (410 mg)	% of Dry cell wt (229 g)
Ajmalicine (**3**)	17	4.0	<0.01
Vallesiachotamine (**4**)	17	4.2	<0.01
Vindolinine (**9**)	0.3	0.1	<0.001
Horhammericine (**7**)	4	0.9	<0.01
Horhammerinine (**8**)	2	0.5	<0.01
Lochnericine (**14**)	2.5	0.6	<0.01
Lochnerinine (**15**)	2	0.5	<0.01
Strictosidine (**12**)	350	83.0	0.15
Strictosidine Lactam (**11**)	30	6.2	0.01

A third line derived from a plant coded 340Y gave the following alkaloids (see table 3): ajmalicine (**3**) (30 mgs, 8.5% of total alkaloid content), vindolinine (**9**) (5 mgs, 1.4%), 19-epivindolinine (**10**) (3 mgs, 0.84%), horhammericine (**7**) (7 mgs, 2%), horhammerinine (**8**) (3 mgs, 0.84%) and *N*,*N*-dimethyltryptamine (**13**) (15 mgs, 4.2%).

TABLE 3. Alkaloids isolated from the 340Y cell line

Alkaloid	Wt (mg)	% of Total alkaloid content[a] (355 mg)	% of Dry cell wt (95.6 g)
Ajmalicine (**3**)	30	8.5	0.03
Vindolinine (**9**)	5	1.4	0.005
19-Epivindolinine (**10**)	3	0.84	0.003
Horhammericine (**7**)	7	2	0.007
Horhammerinine (**8**)	3	0.84	<0.003
N,*N*-dimethyltryptamine (**13**)	15	4.2	0.157

[a]95.6 g cells gave 355 mgs of crude alkaloid 0.37% of dry cell weight.

While a fourth case, a cell line coded 951G, produced small amounts of ajmalicine (**3**) (5 mgs, 1.8% of total alkaloid content) and strictosidine lactam (**11**) (12 mgs, 4.4%), the other alkaloids present were at too low a concentration to evaluate properly. From these results, it can be seen that cell cultures from different plants, though derived and propagated by the same means, do show different enzymatic pathways, perhaps due to loss of activity in specific enzyme(s). This approach does give potentially useful variations in secondary metabolite production. This and other complementary approaches are currently being exploited to afford cell lines that produce alkaloids of pharmaceutical interest.

EXPERIMENTAL

TISSUE SOURCE.—Each cell line was derived from anther explants from *C. roseus* plants; the details on initiation of callus and cell suspension cultures are described elsewhere (1).

LARGE SCALE CELL SUSPENSION CULTURES.—The following procedure was typical for each cell line:

The inoculum was grown in B5 medium (9) in shake flasks over a period of approximately 4 days. Then 500 ml of this inoculum was added to Zenk's alkaloid production medium (8) contained in a 7.5 liter Microferm bioreactor. The growth of the cultures was allowed to proceed for 2 weeks under agitation at 200 rpm and aeration of 35 ml of air per liter culture per minute and at a temperature of 26°. When the culture was harvested after two weeks and the water was removed by freeze drying, dry cells were obtained.

ALKALOID EXTRACTION AND SEPARATION

176G.—Freeze dried cells (62.1 g) were suspended in methanol (300 ml) and extracted in an ultrasonic bath for 4 hr. The suspension was filtered, and the solid was re-extracted (2 x 500 ml). The extracts were combined and concentrated *in vacuo*. The residue was suspended in 1N HCl (150 ml) and washed with ethyl acetate (4 x 50 ml). The aqueous solution was neutralized ($NaHCO_3$), adjusted to a pH of *ca* 9.5 (1N NaOH), and extracted with ethyl acetate (4 x 50 ml). When dried and concentrated the combined extracts afforded the crude alkaloids (270 mg).

This standard extraction procedure was used for each batch of freeze dried cells.

ALKALOID ISOLATION.—This was performed in a routine manner by chromatography of the crude alkaloids over Porasil B (150 g, Waters Associates) in a stainless steel column (30 x 2.5 cm) with H_2O/CH_3CN (68/32) containing 0.1% Et_3N modifier at a flow rate 18 ml/min. A total of 41 x 25 ml fractions were collected then eluted with CH_3CN (100 ml). The 41 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 26–32 were combined and concentrated to give a yellow foam (35 mg). Purification by preparative tlc afforded: yohimbine (**5**) (2.5 mg) identical by mp, ms, uv, ir, nmr, co-tlc and co-hplc with an authentic sample; isositsirikine (**6**) (2 mg) identical to an authentic sample; horhammericine (**7**) (5 mg) identical to an authentic sample; horhammerinine (**8**) (3 mg)

identical to an authentic sample; vindolinine (**9**) (6 mg) and 19-epivindolinine (**10**) (5.5 mg), both identical with authentic samples.

Fraction 41 consisted of two major products. One of these was identified as ajmalicine (**3**) (25 mg) by direct comparison with an authentic sample. The other component (16 mg), mp 187–191°, was assigned on the basis of its spectral data as lochnericine (**14**); this was then confirmed by comparison with an authentic sample.

The other fractions contained numerous alkaloids, but in such minor amounts that it was not possible to fully characterize the component and definitely assign a structure.

Cell line 299Y.—Freeze-dried cells (229 g), when cultivated and worked up as described above, afforded the crude alkaloids (470 mg). Trituration of this crude alkaloid with ether gave an ether soluble mixture (60 mgs) and a residue (410 mgs). This residue was found to be predominantly composed of two compounds which were isolated by preparative tlc. One was identified as strictosidine (**12**) (350 mg) by comparison with authentic material and conversion to its pentaacetate and its lactam. The other was identified as strictosidine lactam (**11**) (30 mg) by comparison with an authentic sample and conversion to its tetraacetate. Separation of the ether soluble mixture, in the same manner as described above, gave ajmalicine (**3**) (16 mg), vallesiachotamine (**4**) (17 mg) identical to an authentic sample, vindolinine (**9**) (0.3 mg), horhammericine (**10**) (2 mg), lochnericine (**14**) (2.5 mg) and lochnerinine (**15**) (2 mg) identified by comparison of its physical and spectral data with that of an authentic sample.

Cell line 340Y.—The freeze dried cells (95.6 g), after extraction, afforded the crude alkaloid mixture (355 mg, 0.37% yield of dry cell weight). Again, the same alkaloid isolation technique described earlier was used to assign the structures of the following components by comparison with authentic samples: ajmalicine (**3**) (30 mg), vindolinine (**9**) (5 mg), 19-epivindolinine (**10**) (3 mg), horhammericine (**7**) (7 mg), horhammerinine (**8**) (3 mg) and *N*,*N*-dimethyltryptamine (**13**) (15 mg).

Cell line 951G.—The crude alkaloid mixture (271 mg) was extracted from the freeze dried cells as above. However, only two components could be categorically assigned a structure; these were ajmalicine (**3**) (5 mg) and strictosidine lactam (**11**) (12 mg).

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