STUDIES ON PLANT TISSUE CULTURES*

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Abstract - A summary of recent experiments with various plant tissue cultures at the University of British Columbia and at the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, is provided. The main emphasis of the research program is to propagate tissue cultures from plant species known to produce active agents of interest in the cancer area.

It is well established that higher plants provide a fertile source of important medicinal agents. In many instances, however, these plants are not readily accessible due to geographical location or alternatively the yield of the desired natural product is extremely low, subject to seasonal variation etc. Attempts to alleviate these situations have led numerous laboratories to consider studies with tissue cultures derived from such plants. Successful research in these directions would clearly provide methodology for a controlled and hopefully reproducible laboratory source for such compounds.

Plant tissue cultures have also been employed in biosynthetic investigations where incorporation levels of proposed precursors are generally higher than in the living plant and they also provide potentially important media for biotransformation studies. A number of books[1-4] and recent review articles[5,6] provide excellent summaries of the previous studies in the tissue culture area. The purpose of this article is not to provide yet another review on the subject but rather to summarize the most recent experiments which have been completed in our program on tissue cultures generated from a variety of plant species which have produced interesting compounds of importance in the cancer area.

*This article is dedicated to Professor Kametani on his retirement from the Chair of Organic Chemistry at the Pharmaceutical Institute of Tohoku University. This author extends to Kamesan, a long time friend and distinguished scientist, sincere wishes for a long and successful retirement.

The specific experiments which we have conducted concern the following plant species: 1) Catharanthus roseus L. G. Don; 2) Maytenus buchananii; 3) Tripterygium wilfordii, and the following discussion summarizes our results within these areas.

1) Studies in Catharanthus roseus

A) Results from other laboratories

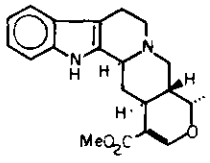
Prior to discussion of our results it is appropriate to briefly summarize the contributions of other groups particularly as they relate to our studies.

Cell cultures of C. roseus have been available for many years and numerous claims of alkaloid production have been made in the literature. Unfortunately some of the earlier experiments have relied entirely on thin layer chromatographic (tlc) analysis rather than actual isolation and characterization of the alkaloids formed by appropriate comparisons with authentic samples. Such data should be regarded as tentative since the complexity of such systems, their respective retention times and/or colour reactions on chromatoplates can lead to misleading conclusions.

The following discussion attempts to summarize the reported presence of alkaloids in callus and/or suspension cell cultures. The compounds have been classified into the main families of indole alkaloids: (i) Corynanthé, (ii) Strychnos, (iii) Aspidosperma, (iv) Iboga.

(i) Corynanthé alkaloids

Ajmalicine (1), an alkaloid with hypotensive activity, and its structural relative, serpentine (2), have been reported from various laboratories.



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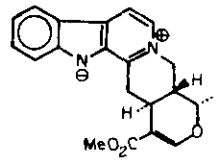

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Babcock and Carew[7] first reported the presence of alkaloids in C. roseus callus tissue cultures while subsequent experiments[8] in both callus and suspension cultures revealed similar occurrences although no identification of alkaloids was made at this time. Patterson[9] and Carew[10] then reported tlc evidence for ajmalicine (1) in their studies with callus and suspension cultures. The calli were initiated from leaves and seedlings while suspension cultures were maintained on a modified Wood-Braun medium.

Reinhard and his colleagues[11] reported low yields of (1) in five different cell cultures from C. roseus tissue while Roller[12] has studied the relative content of (1) and (2) in plants as compared with that observed in callus cultures.

A study on the influence of various media on the culture production of (1) has been reported by Doller[13].

A very detailed investigation of various culture growth conditions for the production of ajmalicine has been reported by Zenk and coworkers[14,15]. These studies revealed a production of (1) in 0.075% yield (based on cell dry weight) and a series of elegant experiments involving enzymes responsible for the synthesis of this alkaloid are also published by this group.

Scott and coworkers[16] have isolated ajmalicine from a "CRW" strain of C. roseus cell cultures grown in SH medium.

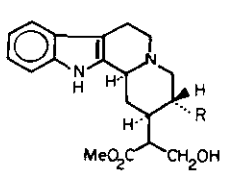
Serpentine (2) has been produced in yields up to 0.8% of cell dry weight in studies by Zenk and coworkers[14,15,17] while Reinhard et al[11] report production of (2) in 0.5% yield based on cell dry weight.

As noted earlier, the studies of Doller[13] have been directed toward the production of (2) in cell suspension and larger scale fermentation experiments.

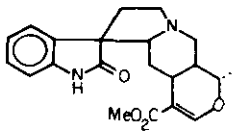
Carew[11] had earlier reported the presence of serpentine (tlc) in callus tissue.

A very recent study[18] involving temperature variations (16-40°C) in the growth of C. roseus suspension cultures has been reported. It was interesting to note that production of (1) and (2) increased with a decrease in the culture temperature.

The Corynanthé alkaloids sitsirikine (3) and dihydrositsirikine (4) have been claimed in callus and suspension cultures[11]. Again in this study, employing suspension cultures grown in modified Wood-Braun medium, identification of these alkaloids was achieved by thin layer chromatography.



3, R = CH=CH$_2$
4, R = CH$_2$CH$_3$



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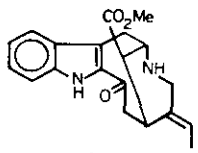

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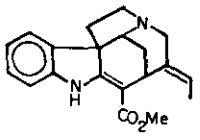
The oxindole alkaloid, mitraphylline (5), biogenetically related to ajmalicine, has been detected (tlc) by Carew[11] in cell suspension cultures, while studies with callus tissue from C. roseus leaf explants[11] report the presence of the 2-acylindole alkaloid perivine (6).

(ii) Strychnos alkaloids

Several reports on the occurrence of members within the Strychnos family have been published. Patterson[9] has detected (tlc) akuammicine (7) in his studies while Scott[16] has reported the isolation of (7) (0.000025% yield) from their "CRW" strain grown in a bioreactor and employing SH medium.



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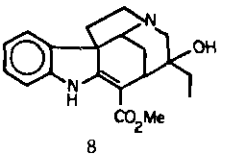

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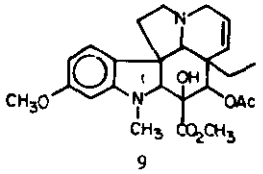
Carew[11] has claimed the presence (tlc) of lochneridine (8) in cultures from C. roseus.

(iii) Aspidosperma alkaloids

Carew and his colleagues[8] have reported the presence (tlc) of the highly oxygenated alkaloid vindoline (9).

A report from the Lilly group[19] employing C. roseus crown gall tissue grown in the dark on a solid medium, and in suspension cultures grown in White's medium in the light, reveals the presence of (9).

A German group[20] cultivated C. roseus callus culture in the dark at 25°C and reported the occurrence (tlc) of vindoline.



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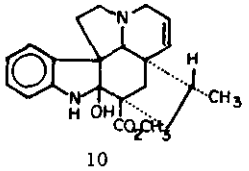

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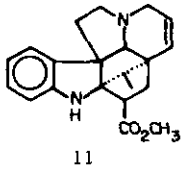

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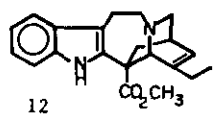
In a most recent study and employing the highly sensitive radioimmunoassay technique, Zenk and coworkers[21] have determined the presence of vindoline (0.002%) in callus cultures from C. roseus leaves.

A report from the Lilly study[19] claims an indication of a "vincoline-like" material (10) in extracts of calli from C. roseus crown gall tissue.

Thin layer chromatography evidence for the presence of the Aspidosperma alkaloid, vindolinine (11) has been reported from extracts of callus tissue grown in the dark for 6 months[20].

(iv) Iboga alkaloids

Much effort has been expended in various laboratories in an attempt to obtain this important class of indole alkaloids. In particular, catharanthine (12), one of the major components in C. roseus and an important unit in the synthesis of bisindole alkaloids (see later), is a prime objective. Thus far only two reports have appeared in which (12) has been substantiated. Scott and coworkers[16] succeeded in obtaining this alkaloid in their "CRW" strain of C. roseus cells and our recent work (see later) has also provided (12) in 0.005% yield.



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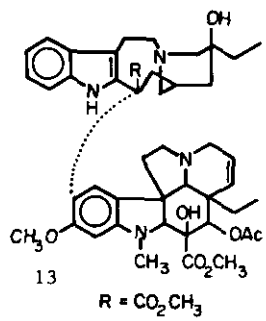
B) Results of our studies

While the above-mentioned investigations were underway in the various laboratories, we initiated a collaborative program between our group at the University of British Columbia and the group at the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon.

The direction of our program was influenced considerably by our earlier studies on the synthesis and biosynthesis of bisindole alkaloids within the vinblastine family. Vinblastine (13) one of



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R = CO_2CH_3

the clinically important anti-tumor agents isolated from C. roseus, represents an important member of these complex natural products and synthetic routes to (13) from more readily available starting materials have been under study for some years. The development of the "biogenetic" approach in our[22] and other[23] laboratories and involving the coupling of catharanthine N-oxide (14) with vindoline afforded an important route to the bisindole system. Figure 2 summarizes our initial studies in which the three bisindole products (15) - (17) were isolated. Under optimum conditions, 3',4'-dehydrovinblastine (15) was obtained in respectable yield (>60%) and its role as an intermediate toward a variety of bisindole alkaloids and derivatives is established[24-27].

In a parallel study in our laboratory[28-30], and utilizing cell free extracts from C. roseus, we were able to demonstrate that 3',4'-dehydrovinblastine (15) is also formed enzymatically from catharanthine (12) and vindoline (9) (Figure 3) and that (15) is subsequently transformed to the alkaloids vinblastine (13), leurosine (18) and catharine (19) (Figure 4).

An independent and simultaneous study by Scott[31] provided results analogous to those outlined in Figure 3.

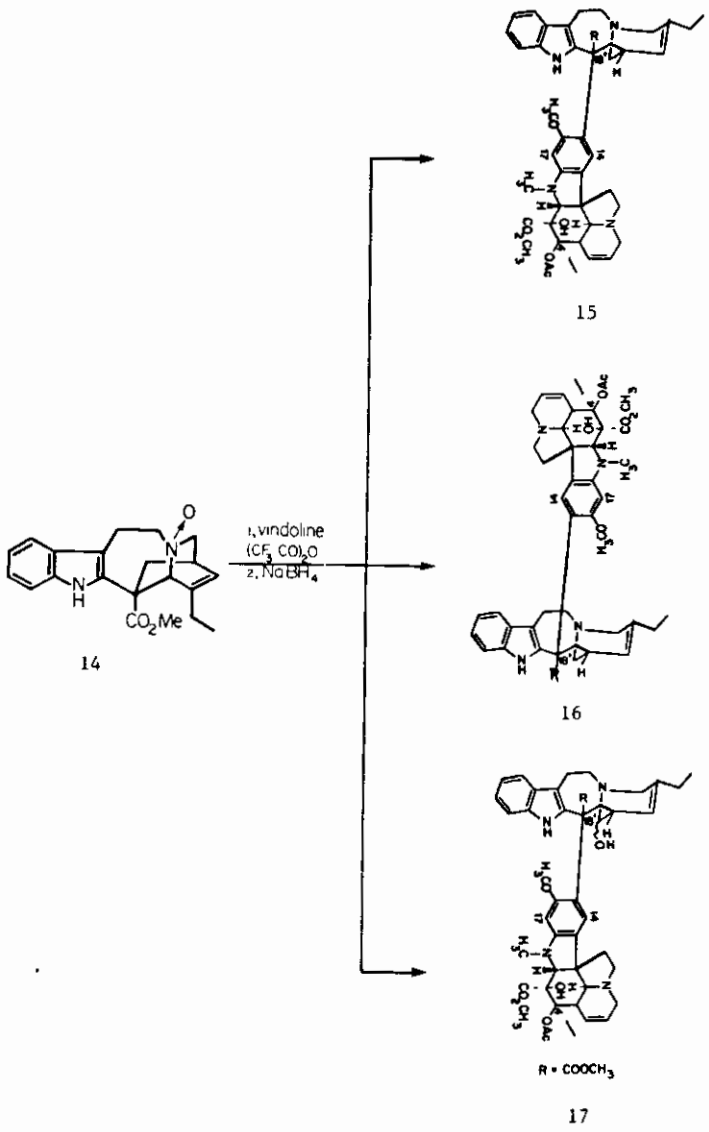


Figure 2. The coupling of catharanthine N-oxide (14) with vindoline.

The above studies clearly demonstrated the importance of the two monomeric alkaloids, vindoline and catharanthine, and these compounds became prime targets in our tissue culture studies. The following discussion summarizes our experiments in this area.

Our initial study[32] was undertaken to delineate the variability of serially cultured callus and cell suspension cultures derived from highly uniform explants, i.e. anthers of buds identical in developmental stage. The only variables introduced were the use of 3 periwinkle cultivars and treatment of buds with a mutagen. In a supplementary study the synthesis and accumulation of alkaloids was related to the growth of those periwinkle cultures which were selected for parti-

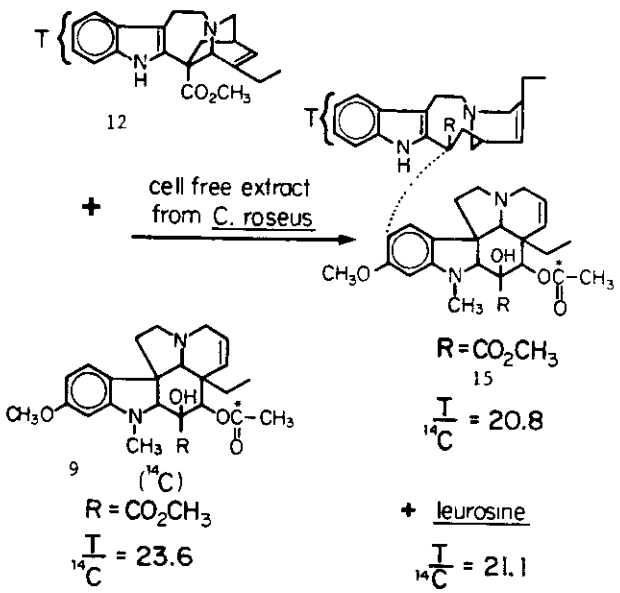


Figure 3. The biosynthesis of 3',4'-dehydrovinblastine (15) and leurosine (18).

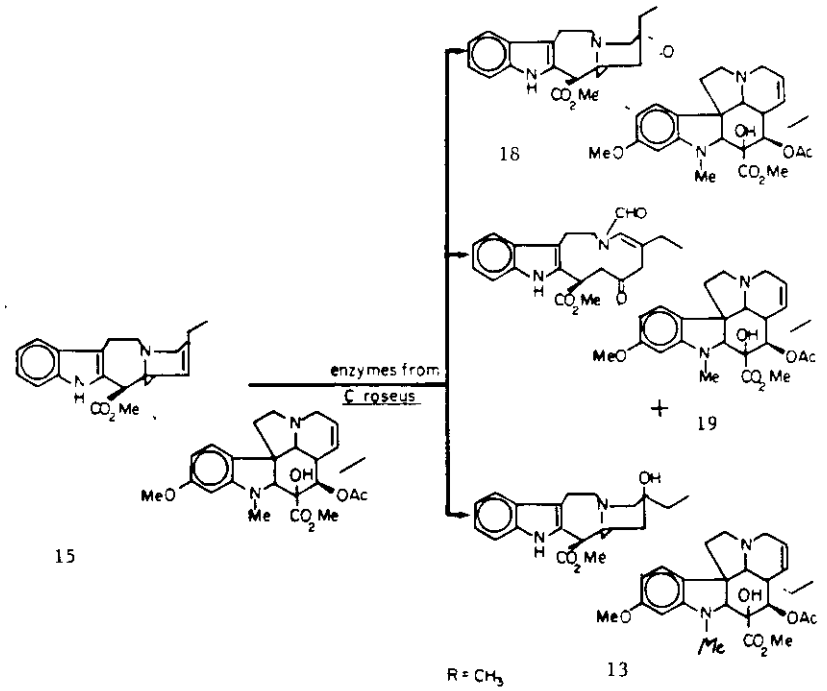


Figure 4. The biosynthesis of vinblastine (13), leurosine (18) and catharine (19) from (15).

cular alkaloid content .

Callus grown from anthers generally originated at the cut of the filament and in the anther walls, i.e. diploid tissue. When grown to a size of 1 - 2 g freshweight, about 2 cm in diameter, the callus was cut into small pieces and serially subcultured on fresh agar medium or transferred to liquid medium (Gamborg's B5 medium) giving rise to a cell suspension. For large scale production Zenk's alkaloid production medium was employed.

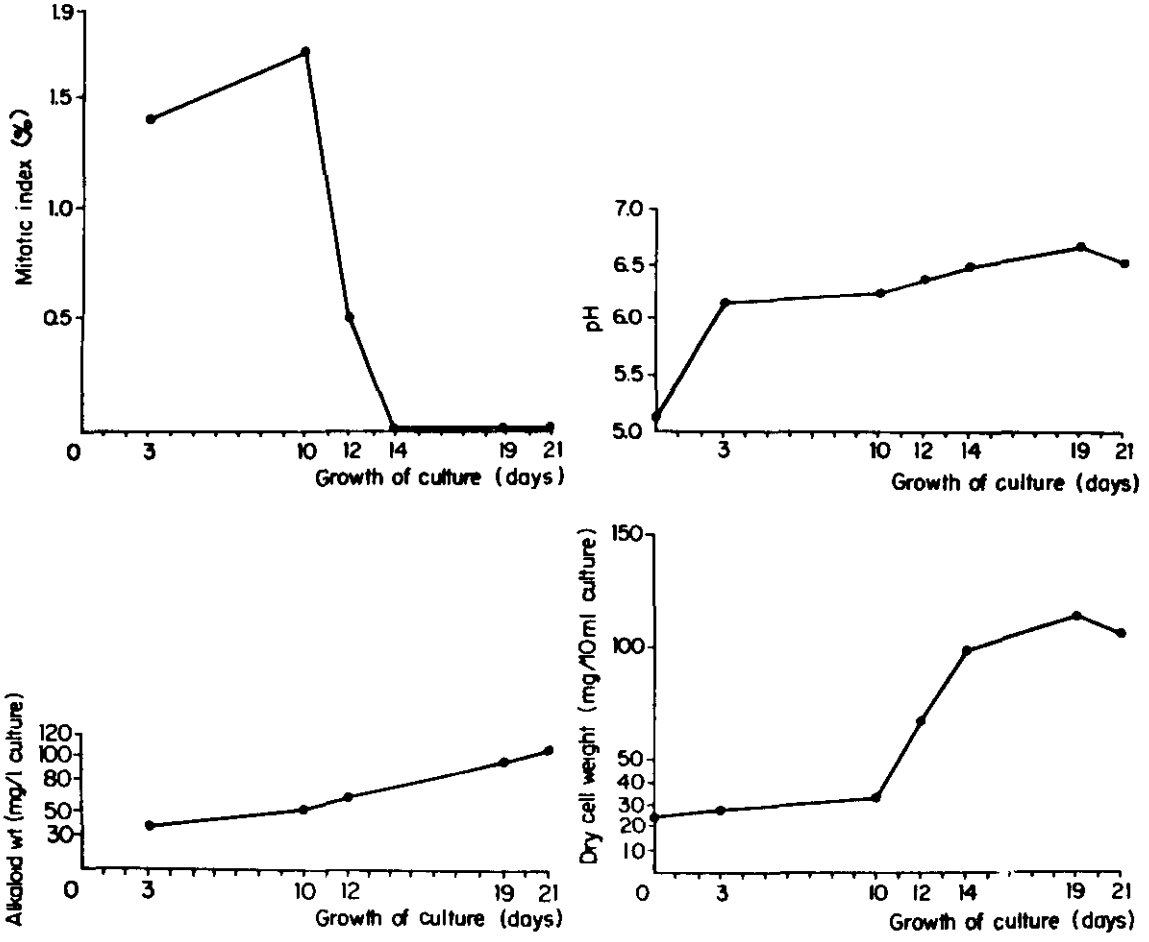
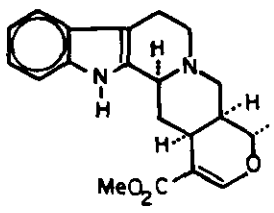


Figure 5.

Cell suspensions generally were greenish, sometimes clumpy cultures. A few serially cultured suspensions, i.e. cell lines, were white and grew at a rate which required transfer to fresh medium twice a week in order to prevent ageing. These cultures consisted of small cell aggregates and single cells. Each culture was variable to the extent that the cells varied in diameter, large ones assuming the dimensions of laticifers (>100 µm in length). The cultures contained 70 - 80% diploid cells, the rest was polyploid, a few giant cells were highly polyploid.

The alkaloid production varied with the cell line and age of the subculture and ranged from 0.1 - 1.5% of cell dryweight. The relative amounts of alkaloids produced was fairly constant under conditions given and appeared cell line specific.

All subcultures of cell lines grown in 7.5 liter Microferm bioreactors followed essentially the pattern shown in Figure 5. After incubation with actively growing cell suspension the mitotic index (MI) dropped to zero within 24 hours and remained there for 2 to 3 days. Thereafter the index rose sharply and reached its maximum (MI 1.8 - 3.0) within 2 days and declined again gradually over the following 10 - 15 days to zero. The cell dryweight over the culture period increased by a factor of 8 to 10 while the variation in pH stayed within half a unit.



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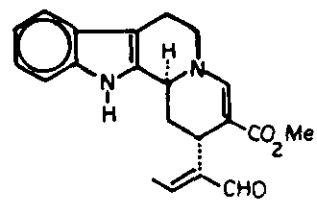

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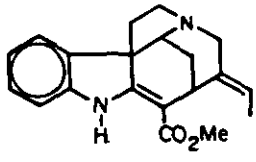

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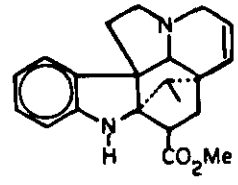

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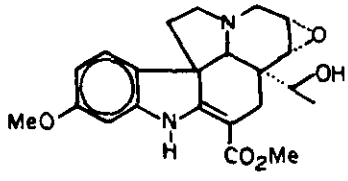

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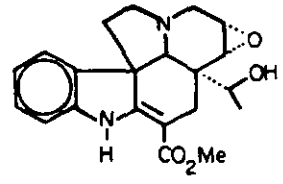

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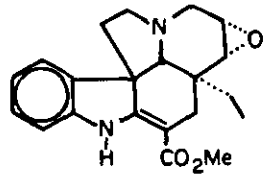

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Analysis of 458 cell lines revealed 312 lines to accumulate alkaloids. Alkaloids identified were Ajmalicine (1), Vallesiachotamine (20), (Corynanthé); Akuammicine (7, Strychnos); and Vindolinine (11), Hörhammerinine (21), Hörhammericine (22) and Lochnericine (23) (Aspidosperma).

It should be noted that (20), (21), (22) and (23) have not been reported in any of the above-mentioned studies.

In general, the alkaloids occurred in a variety of combinations. It was of interest that the combinations were not random but certain combinations appeared at a higher frequency than others. For example 6.73% had Corynanthe-Strychnos alkaloids only; 13.78%, Strychnos-Aspidosperma only; 23.07%, Corynanthe-Aspidosperma alkaloids only, while 13.14% contained all three types of alkaloids. It should be noted that 9.6% had only Corynanthe alkaloids; 13.78% only the Strychnos type and 10.53% contained only the Aspidosperma type. Of the 312 lines producing alkaloids, a total of 76.6% were capable of accumulating Aspidosperma-alkaloids and 46.15% Strychnos while 56.71% yielded Corynanthe type alkaloids.

During an 8 week culture period alkaloids have been found as soon as 2 weeks after inoculation. Most cell lines showed a maximum accumulation of alkaloids in the 3rd - 5th week of culture. Having established a large number of cell lines capable of alkaloid production we proceeded to a more detailed study with several of the more promising lines. The results from two such lines coded as "943" and "200GW" are summarized below.

The 943 line[33]

Studies with this selected cell line were performed both in shake flasks and bioreactors. For the former experiments, callus tissue was added to Erlenmeyer flasks containing LB5 medium and shaken for 5 days (130 rpm) at 27°C in continuous light. The resulting cell suspension was transferred into Erlenmeyers containing Zenk's alkaloid production medium and agitation continued for a further 3 weeks. The alkaloids were subsequently extracted from the freeze-dried cells. For the bioreactor experiments inoculum grown in LB5 medium as above was transferred to the bioreactor containing Zenk's alkaloid production medium. Under agitation at 200 rpm, aeration of 35 ml of air per liter culture per minute and a temperature of 26°C, growth was allowed to proceed for 2 weeks. Samples were withdrawn at various time intervals and analyses of mitotic index (MI), dry cell weight and pH were made (Figure 5). Here again alkaloids are found in the cells. To illustrate some comparison between the various culture growths in shake flasks and bioreactors the alkaloid content of various samples of the 943 cell line is summarized in Table 1.

We have found that alkaloid content is optimum after the mitotic index has decreased to zero and if no addition of nutrients is made during the course of the culture growth. Sample 2 (Table 1) illustrates the detrimental effect of adding tryptophan after fermentation has proceeded for some time. Presumably the addition of this amino acid provides a nutrient which stimulates cell division and thus rapid catabolism of alkaloids.

Analysis of the alkaloid mixture was achieved by high pressure liquid chromatography (HPLC) em-

ploying the Waters Radial Compression Module fitted with a reverse phase pack and dual channel detection at 254 and 280 nm. Figures 6 and 7 illustrate traces for the 943 cell line grown in shake flasks (Sample No. 5, Table 1) and bioreactor (Sample No. 3, Table 1) respectively.

Table 1

Alkaloid Content of the 943 Cell Line

Sample	Cell-dry wt. (g)	Alkaloids as % of Dry Cell Wt.	Growth Period (weeks)	Culture Type
1	7.8	0.44	3	Microferm bioreactor
2*	114	0.11	3 + 1 day	" "
3	145	0.19	2	" "
4	95	0.39	3	Shake flasks
5	120	0.25	3	" "

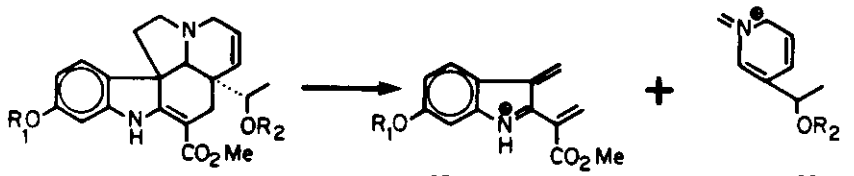
* Tryptophan (100 mg/liter) was added to the same fermentation as sample 1 and harvesting occurred 1 day later.

Table 2 illustrates the percentage of the various alkaloids present in mixtures obtained in the shake flask and bioreactor experiments as shown in the HPLC analyses given in Figures 6 and 7. In addition to the known alkaloids discussed above (Table 2), four others, previously not found in other tissue culture studies were isolated. Three of these could be assigned the structures: 19-acetoxy-11-methoxytabersonine (24), 19-hydroxy-11-methoxytabersonine (or vandrikidine)[34] and 19-acetoxy-11-hydroxytabersonine (26) on the basis of their characteristic spectroscopic data and chemical inter-relations.

For example, all three compounds displayed the same mass spectral fragmentation pattern, though of course individual ions varied according to the different substituents. The major ions (27 and 28) indicated that all three possessed oxygen substituents in the indole nucleus and in the side chain.

Since no direct comparison with authentic material was possible, their spectral data was compared with that of the known 19-hydroxytabersonine, prepared from vindolinine[35]. These comparisons confirmed the assigned structures.

The interrelationships between these compounds were confirmed by chemical correlation. Treatment of (25) with acetic anhydride/pyridine afforded (24) as did methylation of (26) with diazo-



27 28

	R_1	R_2	M^+	R_1	m/e	R_2	m/e
24	Me	Ac	424	Me	244	Ac	193
25	Me	H	382	Me	244	H	151
26	H	Ac	410	H	214	Ac	193

methane.

A fourth very polar compound was isolated from the methanol soluble alkaloid fraction and assigned the novel β-glycoside structure (29). Its ultraviolet spectrum with maxima at 290 and 327 nm,

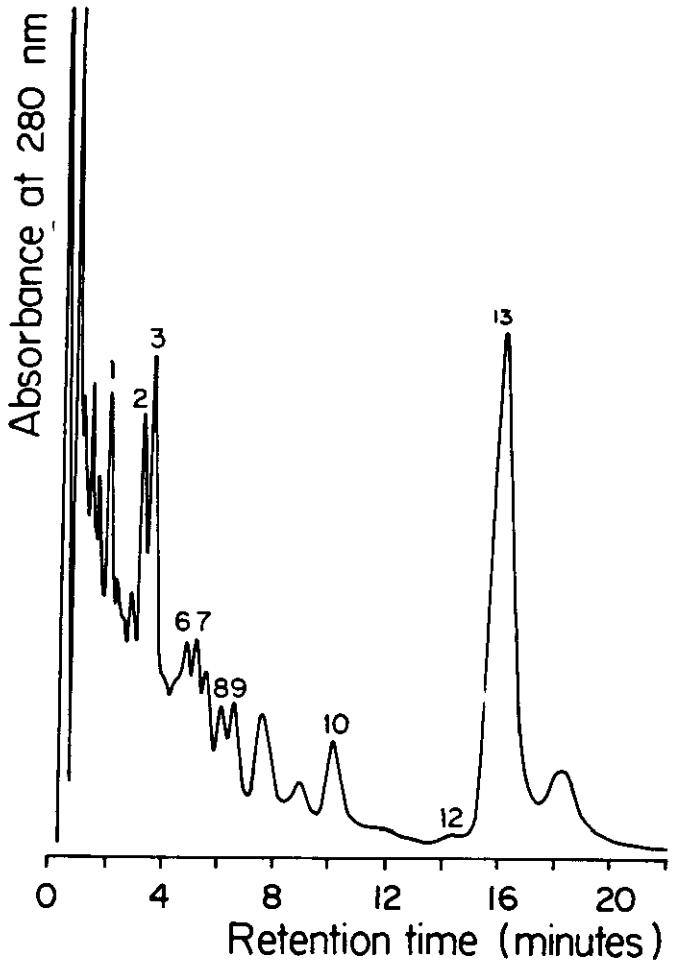
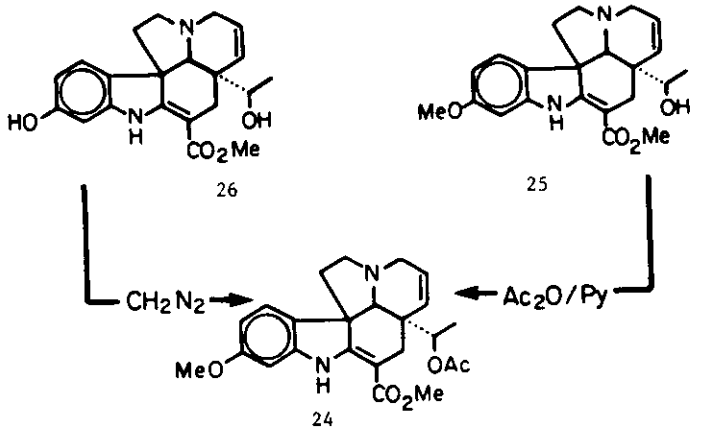


Figure 6



together with infrared absorption bands at 1602 and 1665 cm^{-1} suggested the enamino-ester function noted above in the alkaloids (24 - 26) and in the Strychnos alkaloid akuammicine (7). The mass spectrum of (29) and several of its derivatives (30 and 31) revealed an ion at m/e 121 (32) gener-

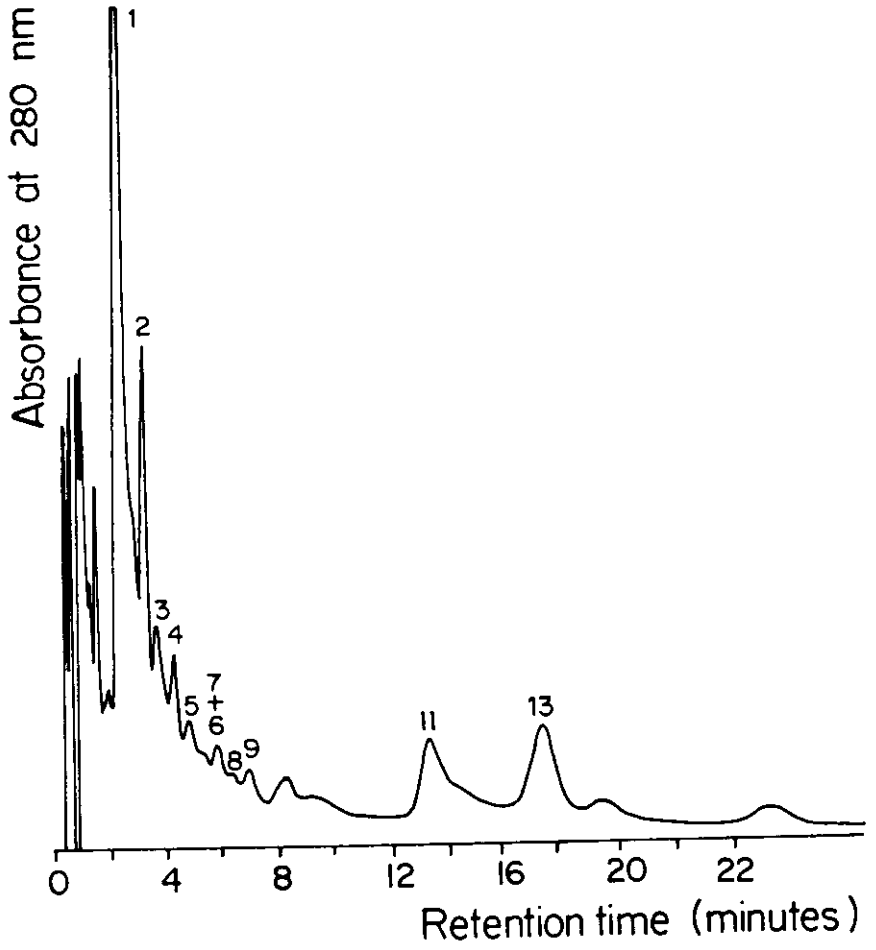


Figure 7

Table 2

Peak No. (Figs. 6 and 7)	Alkaloid	% of alkaloid in crude sample†	
		Sample 5, Table 1 (Fig. 6)	Sample 3, Table 1 (Fig. 7)
1	Glycoside (29)	8.8	27.6
2	Unknown	5.2	7.5
3	Unknown	9.8	3.5
4	Yohimbine	-	4.6
5	Isositsirikine	-	4.5
6	Hörhammericine	5.06	3.0
7	Hörhammerinine	3.15	
8	19-epivindolinine	2.7	1.5
9	Vindolinine	4.7	2.5
10	Unknown	3.0	
11	Dimethyltryptamine	-	4.11
12	Vallesiachotamine	0.12	0.52
13	Ajmalicine	18.94	7.74

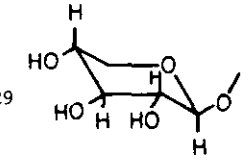
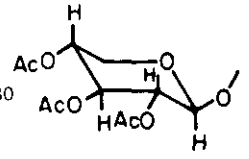
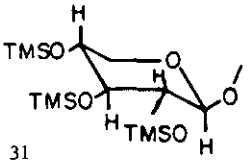
† Based on relative absorbance at 280 nm.

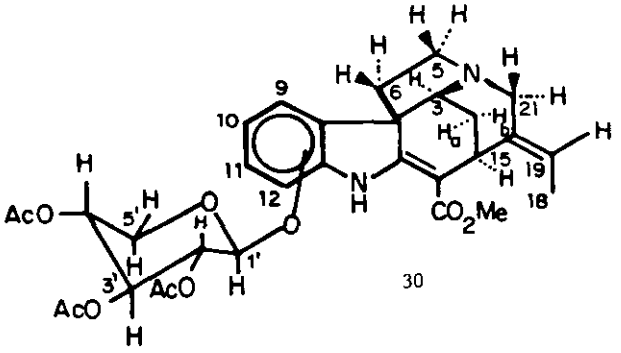
ally considered characteristic of the Strychnos series and thus favored placing this new alkaloid within this family. The molecular ion of (29) occurred at m/e 470 and a significant ion at m/e 338 suggested loss of $C_5H_8O_4$, the latter being possibly attributed to a C_5-carbohydrate unit. Confirmation of the latter could be obtained from acetylation of (29) which provided a triacetate 30 (m/e 596 (M^+), $C_{31}H_{36}N_2O_{10}$) in which again loss of the acetylated carbohydrate unit (m/e 338, M^+-258) could be seen in the mass spectrometer. In similar fashion the trimethylsilyl ether derivative (31) revealed an ion at m/e 338 corresponding to the expected loss from the molecular ion.

Of particular importance were the very instructive NMR spectra (determined at 270 MHz) of (29) and its derivatives. The triacetate (30) revealed a series of well separated proton signals and by appropriate decoupling experiments all the protons in the molecule could be assigned. The only ambiguity in this novel structure is the attachment (C_9 or C_{12}) of the xylose unit since NMR data

did not allow a clear differentiation between these alternatives.

32

	R	M^+	M^+–sugar	m/e
7	H	322		121
29		470	338	121
30		596	338	121
31		686	338	121

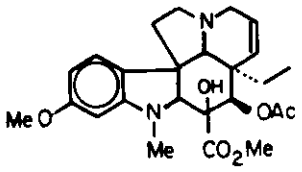


30

It is clear that the 943 cell line provides a rather broad spectrum of alkaloids with representatives from the Corynanthé, Strychnos and Aspidosperma families. Members of the Iboga family were however, not observed during these studies. The possibility that such systems are present cannot be excluded since a number of very minor components in the mixture remain unidentified.

Each batch of cells was also monitored for the alkaloid vindoline (9), the major component of C. roseus, both by HPLC and radioimmunoassay but no indication of (9) was found.

From a biosynthetic standpoint a possible interrelation between the Aspidosperma alkaloids found



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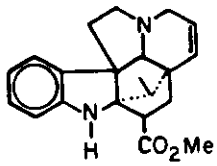


37

in the 943 cell line is suggested in Figure 8. Here activation of the saturated side chain of tabersonine (33) perhaps by enzyme mediated hydrogen abstraction could provide the intermediate (34) which in turn cyclizes to 35 and finally to vindolinine (11) and 19-epivindolinine (37).

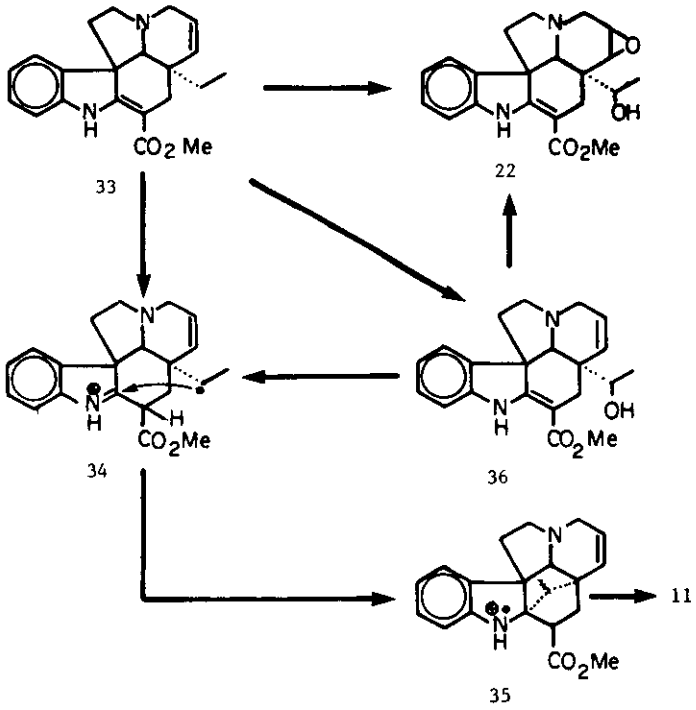


Figure 8

It is also feasible to consider that the free radical intermediate (34) undergoes oxygenation to a peroxide species which on reduction would yield the hydroxy-ethyl side chain observed in one of isolated alkaloids (22) or that some alternative enzyme hydroxylation reaction occurs to yield (36). It is plausible to consider that (36) then undergoes epoxidation to (22). Such biogenetic considerations imply that formation of the isolated alkaloids (22), (11), (37) via enzymic activation of the ethyl side chain in (33) consume the necessary tabersonine system required for elaboration to vindoline (9), the latter bearing additional oxygen functionality in ring C. On this basis it would appear that the 943 cell line lacks the required enzymes for ring C functionalization but yet maintains enzymatic activity associated with side chain functionalization.

It is also noteworthy that none of the alkaloids isolated within the Aspidosperma family contain an N-methyl function present in vindoline and thus the 943 cell line appears to lack this type of methylating enzyme.

The results from a 953 cell line which produces similar alkaloids to those discussed above will be published elsewhere[36] and is not discussed here.

The 200GW line[37]

Another particularly interesting cell line under recent investigation is coded as "200GW". The general procedures concerning tissue propagation, HPLC analyses etc. are very similar to those discussed above. However this line is uniquely different from the 943 line and produces its own "spectrum" of alkaloids as summarized in Table 3 and Figure 9.

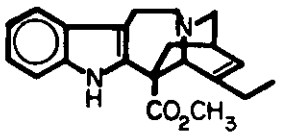
Table 3

Alkaloids isolated from the 200GW Cell Line

Alkaloid	%† Yield from dry cell wt.	%† of crude alkaloid mixture
12	0.005	1.35
20	0.015	4.05
38	0.026	7.02
1	0.006	1.62
22	0.002	0.54
21	0.005	1.44
11	0.002	0.54
37	0.002	0.54
39	0.224	60.48

† figures refer to isolated yields.

In a typical bioreactor experiment, 140 gms of freeze dried cells provide 520 mg of a crude alkaloid mixture (0.37%) which after separation by HPLC provide the alkaloids in the yields shown in Table 3. Of particular interest is the alkaloid catharanthine (12, 7 mg, *0.005% dry cell wt*) isolated for the first time in our studies. The 200GW line provides this alkaloid in amounts ca three times that normally obtainable from C. roseus plant material (generally ca 0.0017% yield from dried plant material). As noted earlier A.I. Scott and his group[16] have *also reported this* alkaloid from callus tissue of C. roseus seedlings and maintained on SH medium. It appears that our line produces (12) in larger amounts.



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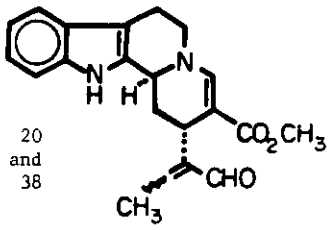

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and
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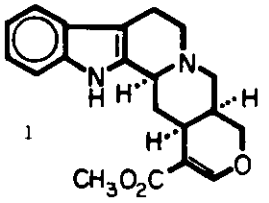

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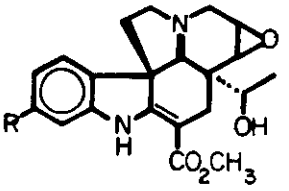
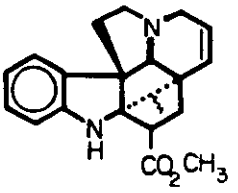


22 R=H
21 R=OCH3



11 and 37

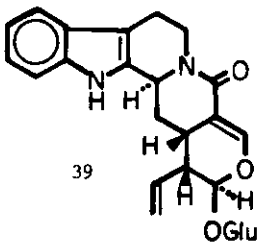


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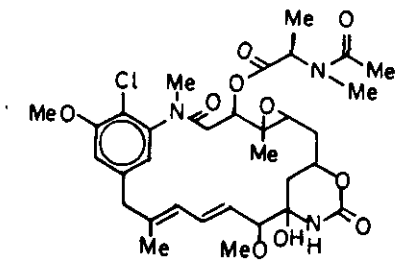
Figure 9. A summary of alkaloids isolated from the 200GW cell line.

Also of interest is the isolation of strictosidine lactam (39), a very major component in the alkaloid mixture produced by the 200GW cell line.

Further studies with these and other lines of C. roseus are underway and will be reported elsewhere.

2) Studies in Maytenus buchananii

Another plant species under recent investigation in our laboratory is Maytenus buchananii (Loes) R. Wilezek, one of the several members of the Celastraceae from which the antitumour agent maytansine (40) has been isolated[38-40]. Our initial studies with plant tissue cultures from the plant have not produced maytansine but several other cytotoxic agents have been isolated and charac-



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terized.

A large number (>200) of explants were cultured onto a wide variety of media. M. buchananii tissues from all sources readily formed calluses on B5, PRL-4, MS and SH basic media, variously supplemented with indoleacetic acid, naphthylacetic acid, 2,4-dichlorophenoxyacetic acid, 4-aminobenzoic acid, kinetin, 4-chlorophenoxyacetic acid, thiamine hydrochloride and coconut water. With the exception of MS medium containing 6×10^{-5} M indoleacetic acid with 1.9×10^{-7} M kinetin, and B5 medium containing no phytohormone supplements, all media and conditions promoted rapid induction and growth of calluses. Calluses appeared to be of heterogeneous composition as indicated by green, black, yellow and orange regions. Many of the calluses bore a profusion of roots.

A number of calluses were chosen for suspension culture. Typically after 3 - 6 weeks, suspensions of mixed aggregate and single cells were produced. The most rapidly growing of these cultures achieved mass doubling times of approximately 3 - 4 days. Cell yields were in the order of 12 - 16 g dry weight of cells per litre of culture.

Extensive thin layer chromatography analysis of both the crude extracts and chromatographic fractions did not reveal the presence of the target compound in any cultures. The TLC assay was shown by use of standard solutions to have a detection limit of 10 ng of maytansine. However bioassay by the KB cell assay[41] showed some of the crude extracts to contain cytotoxic components ($ED_{50} < 5 \times 10^{0}$ μg/ml). This compares with an ED_{50} value of 2×10^{-1} μg/ml for crude plant extracts containing maytansine (see Table 4).

In order to determine the nature of the cytotoxic components of the culture extracts, one suspension culture was scaled up to a 10 l. volume to provide sufficient material for natural product isolation.

For isolation, the crude alcohol extract of the cells was partitioned between ethyl acetate and water, and then petroleum ether and 10% aqueous methanol. β-Sitosterol was the major component of the petroleum ether fraction. Column chromatography followed by preparative layer chromatography of the aqueous methanol phase yielded polpunonic acid (41), tingenone (51) and 22β-hydroxytingenone (52) in yields of 0.043, 0.005 and 0.005% of dry weight of cells respectively. The

Table 4

	ED_{50} †
Maytansine*	2×10^{-5}
Crude plant extracts containing maytansine*	2×10^{-1}
Crude extracts from tissue culture MYT 1e1	3.8×10^{0}
Tingenone	2.7×10^{-1}
22β-hydroxytingenone	2.5×10^{0}

†ED_{50} is expressed in μg/ml and is the calculated effective concentration which inhibits growth of 50% of control growth. Assay was done using KB cells (human epidermoid carcinoma of the nasopharynx).

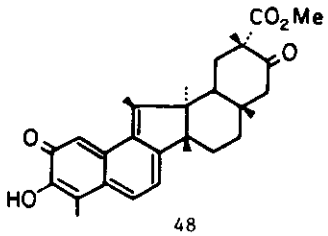
*Data from Dr. M. Suffness, National Cancer Institute, National Institutes of Health.

acid (41) was characterised as its methyl ester (42) and identified by direct comparison with authentic samples. The two orange pigments tingenone and 22β-hydroxytingenone were identified by their mass and PMR spectra[42 43]. Tingenone (51) was also compared with an authentic sample. Tingenone and 22β-hydroxytingenone gave KB assay ED_{50} values of 2.7×10^{-1} and 2.5×10^{0} μg/ml respectively, and these may account for the KB activity of the crude culture extracts.

Partial purification of the more polar components of the culture extract as their methyl esters yielded a complex mixture of triterpene esters which gave six peaks on GLC as their TMSi derivatives. Due to the limited amount of material available at this time further investigations were not pursued.

The presence of tingenone and 22β-hydroxytingenone in tissue cultures of M. buchananii is not surprising as triterpene quinone-methides of this type have previously been isolated from the following members of the Celastraceae:- Celastrus scandens[44] (celastrol (43, R = H), Tripterygium wilfordii[45] (celastrol), Pristimera indica[46] (pristimerin (44), Maytenus chuchuhuasca[47] (pristimerin and tingenone), Maytenus dispermus[48], (pristimerin and dispermoquinone (45)), Euonymus tingens[42,49,50] (tingenone, 22β-hydroxytingenone and 20-hydroxytingenone (46), Maytenus sp.[43] (22β-hydroxytingenone), Maytenus ilicifolia[51] (pristimerin and tingenone), Plenckia polpunea[52] (tingenone), Catha cassinoides[53] (celastrol, tingenone, pristimerin and iguesterin (47)), Salacia macrosperma[54] (pristimerin, tingenone, 20-hydroxytingenone and salacia quinonemethide (48)), Schaefferia cuneifolia[55] (pristimerin and tingenone).

The isolation of maytenonic acid from Maytenus senegalensis has been reported[56,57]. These workers identified this compound as a 3-keto-friedelane bearing a carboxyl group at C-20. The stereochemistry at C-20 was not defined. It seems likely, on chemotaxonomic grounds, that may-



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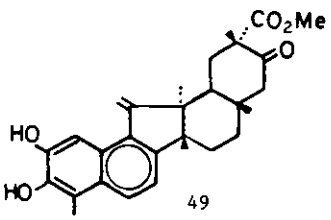

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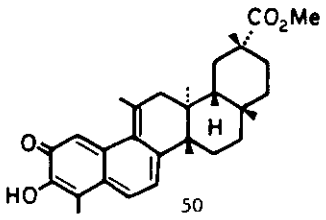

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tenonic acid is the same as polpunonic acid (i.e. 20α -COOH). The co-occurrence of polpunonic acid and tingenone in Pleuckia polpunea[52] and also in the M. buchananii tissue cultures suggests that these compounds are biogenetically related. Viswanathan[58] has reported the isolation of salaspermic acid (50) as well as pristimerin, tingenone and hydroxytingenone from Salacia macrosperma. Again the co-occurrence of these compounds suggest a common biogenesis. Possible biosynthetic relationships between polpunonic acid, salaspermic acid and the triterpene quinone-methides are summarised in Scheme I.

Reddy et al[54] have reported the isolation of salacia quinone-methide from Salacia macrosperma. They have suggested structure (48) for this new quinone-methide on spectroscopic evidence. The structure proposed appears unlikely as it would exist in the more stable aromatic form (49). In fact a very recent paper on new quinone-methides has also expressed doubt as to the correctness of structure (48) and has suggested that it might be identical with the compound pristimerinene (50) isolated from Prionostemma aspera[59].

A detailed publication on our studies with M. buchananii has been submitted[60].

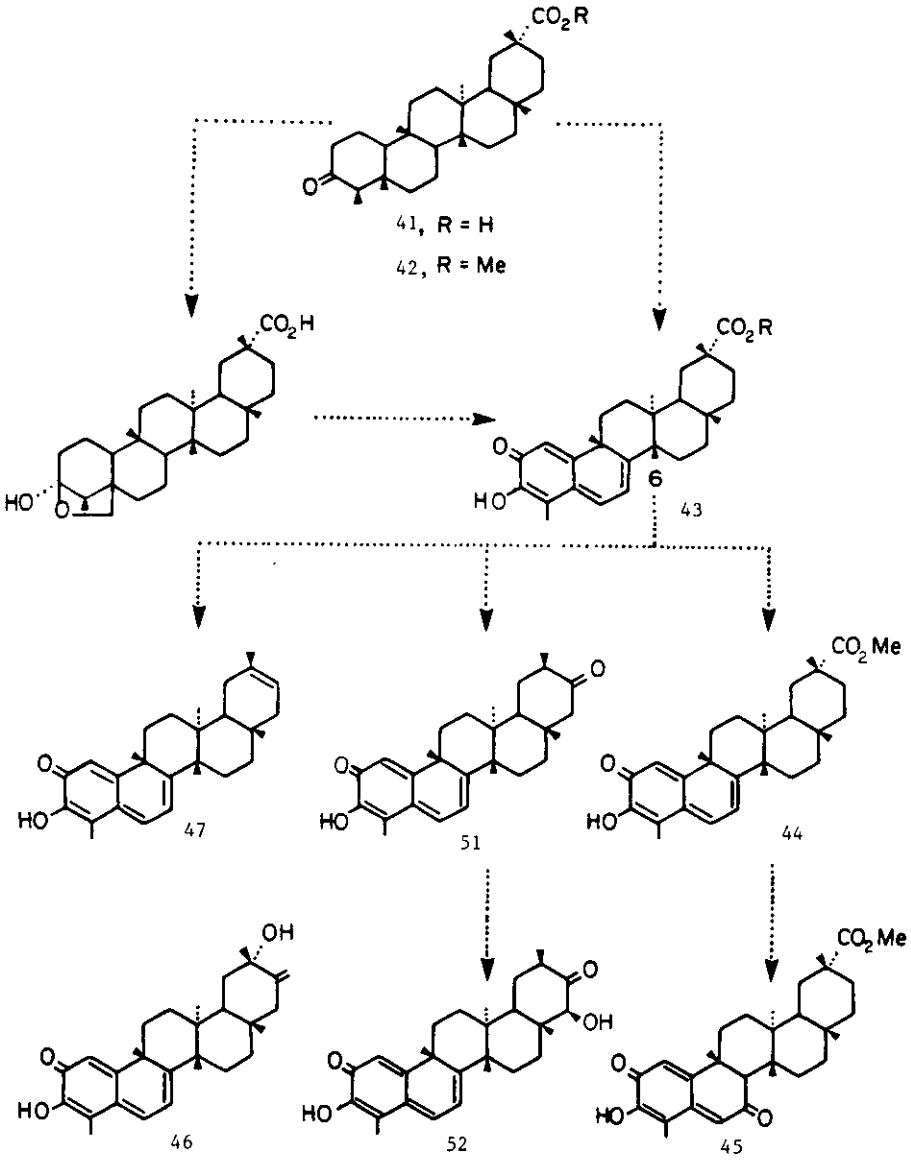
3) Studies with Tripterygium wilfordii

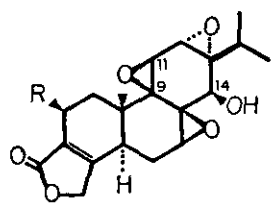
Tripdiolide (53, R = OH) and triptolide (53, R = H) are interesting diterpene triepoxides with significant activity in vivo against L-1210 and P-388 leukemias in the mouse and in vitro against cells derived from human carcinoma of the nasopharynx (KB)[61]. The plant, Tripterygium wilfordii, in which they occur in low concentration (0.001%), is not readily accessible and thus studies with tissue cultures were of interest in our program.

Plant tissue culture cells were grown in callus and in suspension employing modified B-5 and PRL-4 media. Experiments with shake flasks over varying time periods revealed upon subsequent

analysis, the presence of tripdiolide and other components. An extensive study concerned with varying parameters (media, various additives, time of growth etc.) for the culture growth has been undertaken and will be published elsewhere. It is sufficient to mention here that, in general, tripdiolide appears in the culture after several weeks (4 - 7 weeks) and its concentration depends on the media and other parameters. Large scale experiments both in shake flasks and in bioreactors have been completed and an extensive investigation of the natural products present has been undertaken. In addition to tripdiolide, fourteen other compounds have been isolated and their structures are presently under study. Some of these are quinone methides

Scheme I





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similar to those encountered in the above studies with M. buchananii, while others are di- and triterpenoid in nature. At present, celastrol (43, R = H), polpunonic acid (41), oleanolic acid and β-sitosterol have been definitely established by appropriate comparisons with spectral data and authentic samples.

It is important to note that in the present tissue culture studies the concentration of tripdiolide is significantly higher than in the plant and further optimization studies are underway. Our initial experiments in this area will be published shortly[62] while a more detailed publication will be submitted at a later date.

In conclusion we hope that this brief summary of our investigations being pursued in several laboratories at the University of British Columbia and at the NRC Prairie Regional Laboratory in Saskatoon will serve to illustrate our general approach in the plant tissue culture area. We believe that the results illustrate the potential of the utilization of plant tissue culture methods in the production of clinically useful antineoplastic agents. However further experimentation is essential before applications to the commercial production of such compounds can be made.

Acknowledgements:

The author serves merely as a spokesman for the enthusiastic group of researchers who have made possible the above discussion. The studies with C. roseus represent a collaborative program between the author's laboratory and the National Research Council of Canada, Prairie Regional Laboratory in Saskatoon. The senior collaborators in Saskatoon are Drs. W.G.W. Kurz and F. Constabel and they along with their associates, K.B. Chatson, H. Evans, P. Gaudet - La Prairie, S. Rambold and J. Rushkowsky are responsible for all of the development of the cell culture methods and large scale production of the natural products. Development of all the analytical methodology (hplc, radioimmunoassay etc.) and the subsequent isolation and characterization of the various alkaloids discussed was performed in the author's laboratory through the efforts of L.S.L. Choi, P. Kolodziejczyk, S.K. Sleigh, K.L. Stuart and B.R. Worth.

Studies with M. buchananii and T. wilfordii represent a collaborative program between the author's laboratory and that of Prof. P.M. Townsley, Dept. of Food Science at this university. The development of the cell culture methods was performed jointly at Food Science by P.M. Townsley, W.T.

Chalmers, D.J. Donnelly, and K. Nilsson; G.G. Jacoli, Canada Dept. of Agriculture, Vancouver; and by P.J. Salisbury and G. Hewitt at the Chemistry Department. Analytical methods and all subsequent isolation of the natural products were pursued at the Chemistry Department by M.H. Beale, T. Kurihara, R.D. Sindelar, K.L. Stuart and B.R. Worth.

It is a particular pleasure to acknowledge the efforts of these colleagues.

We are grateful to Prof. G.B. Marini-Bettolo, Universita Cattolica, Rome for samples of polpunonic acid, methyl polpunonate and tingenone and to Dr. Mildred Broome, Arthur D. Little and Co., Cambridge, Mass. for the KB assays.

Financial aid at this university for the C. roseus program was provided by a grant to the author from the Natural Sciences and Engineering Research Council of Canada and an NRC Research Contract under the Fermentation Technology Program (00-310-SX-8-3011).

Financial aid for the studies on Maytenus and Tripterygium was provided by the National Institutes of Health (Contract NO1-CM-87236).

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Received, 4th September, 1980