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STUDIES IN PLANT TISSUE CULTURE: POTENTIAL SOURCES OF CLINICALLY IMPORTANT ANTI-TUMOR AGENTS

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Abstract - Tissue culture studies have been conducted on the plant species, Catharanthus roseus, Maytenus buchananii, Tryperygium wilfordii, and Cephalotaxus harringtonia, which produce compounds having anti-tumor properties.

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.

This lecture will 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.

The specific experiments which we have conducted concern the following plant species: 1) Catharanthus roseus L. G. Don; 2) Maytenus buchananii; 3) Tripterygium wilfordii; 4) Cephalotaxus harringtonia and Fig. 1 summarizes the target compounds which are involved in our program.

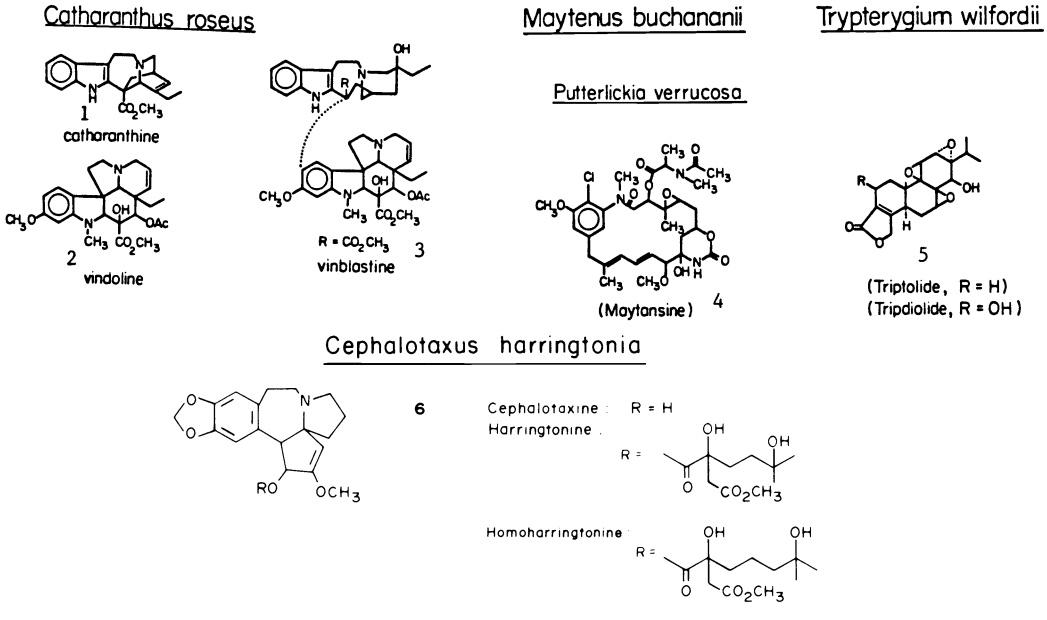


Fig. 1. Plant species under study and the target compounds involved.

1) Studies in Catharanthus roseus

Several years ago 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 (3) one of the clinically important anti-tumor agents isolated from C. roseus, represents an important member of these complex natural products and synthetic routes to (3) from more readily available starting materials have been under study for some years. The development of the "biogenetic" approach in our[7,9-11] and other[8,12] laboratories and involving the coupling of catharanthine N-oxide (7) with vindoline afforded an important route to the bisindole system. Figure 2 summarizes our initial studies in which the three bisindole products (8) - (10) were isolated. Under optimum conditions, 3',4'-dehydrovinblastine (8) was obtained in respectable yield (>60%) and its role as an intermediate toward a variety of bisindole alkaloids and derivatives is established[9-12]. In a parallel study in our laboratory[13-15], and utilizing cell free extracts from C. roseus, we were able to demonstrate that 3',4'-dehydrovinblastine (8) is also formed enzymatically from catharanthine (1) and vindoline (2) (Figure 3) and that (8) is subsequently transformed to the alkaloids vinblastine (13, R = CH_3), leurosine (11) and catharine (12) (Fig. 4).

An independent and simultaneous study by Scott[16] provided results analogous to those outlined in Fig. 3.

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. Details of these studies are available in various recent publications[17-25].

Our initial study[17,18] 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 particular alkaloid content.

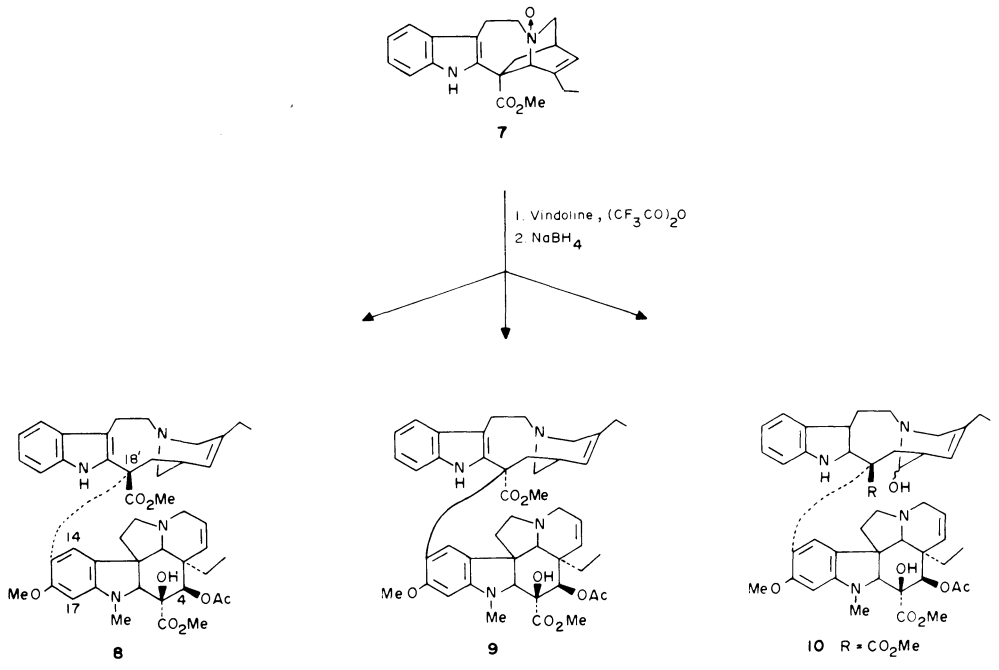


Fig. 2. Summary of results when catharanthine N-oxide (7) is coupled with vindoline (2).

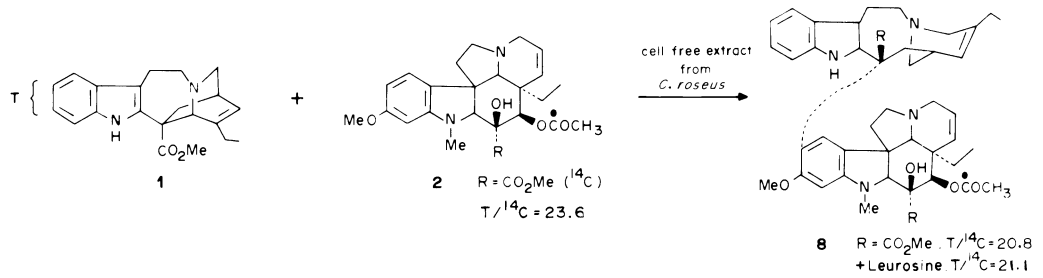


Fig. 3. The biosynthesis of 3',4'-dehydrovinblastine (8) and leurosine (11) from catharanthine (1) and vindoline (2) employing cell free extracts.

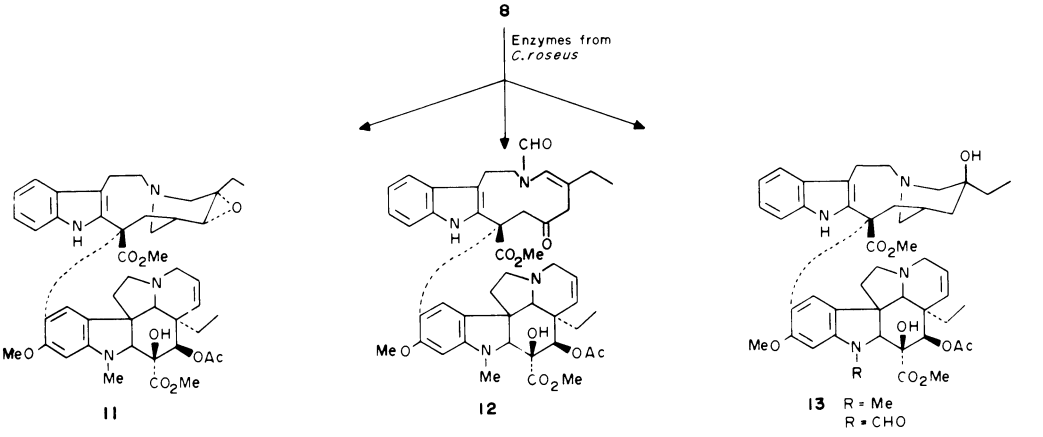


Fig. 4. Enzyme catalyzed conversion of 3',4'-dehydrovinblastine (8) to leurosine (11), catharine (12) and vinblastine (13, R = CH_3) employing cell free extracts.

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.

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 Fig. 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.

Analysis of 458 cell lines revealed 312 lines to accumulate alkaloids belonging to Corynanthé, Strychnos, Aspidosperma and Iboga families.

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 Corynanthé-Strychnos alkaloids only; 13.78%, Strychnos-Aspidosperma only; 23.07%, Corynanthé-Aspidosperma alkaloids only, while 13.14% contained all three types of alkaloids. It should be noted that 9.6% had only Corynanthé 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 Corynanthé type alkaloids. Only several lines produced catharanthine (1), a member of the Iboga family.

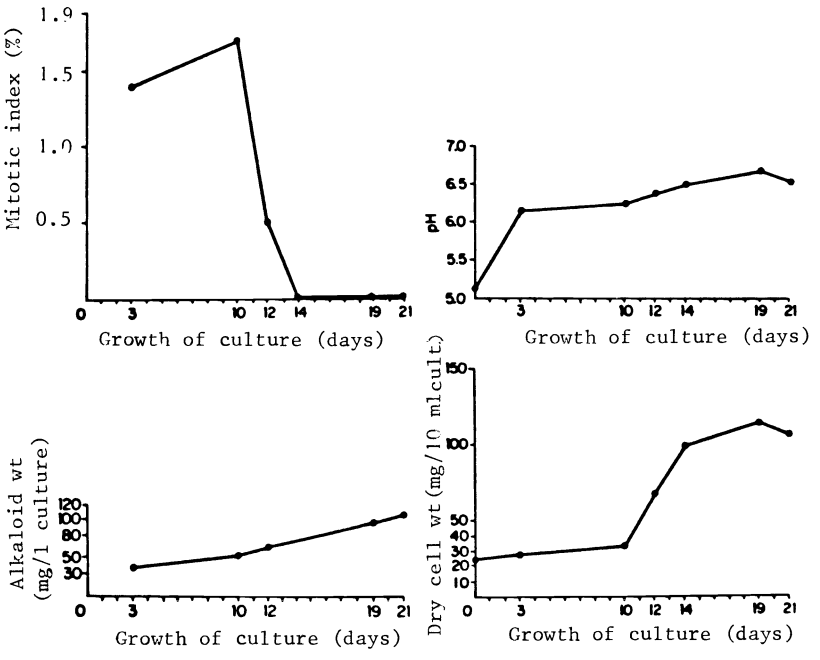


Fig. 5. General growth pattern of C. roseus tissue culture in bioreactor.

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 "953" and "200GW" are summarized below.

a) The 953 line

Studies with this selected line were performed both in shake flasks and bioreactors employing the lB5 medium for inoculum growth and then Zenk's alkaloid production medium. Detailed accounts of these experiments are published[20,23] so only a brief summary is provided. On harvesting the culture, the water is removed by freeze drying and the alkaloids are extracted in the conventional manner to provide the data summarized 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 (14), yohimbine (15), isositsirikine (16), vallesiachotamine (17), strictosidine lactam (18), lochnericine (19), hörhammericine (20), hörhammerinine (21), vindolinine (22), 19-epivindolinine (23), 19-acetoxy-11-methoxytabersonine (24), 19-hydroxy-11-methoxytabersonine (25) and dimethyltryptamine (26).

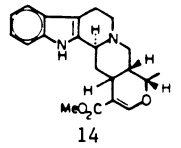
Since general alkaloid formation was not observed during the initial periods of rapid cell growth, it was decided to examine whether the appearance, disappearance or build-up of particular components could be observed over different time periods. The results are given in Tables 1 and 2 and Fig. 6. These show that the percentage of alkaloid per gram of cell weight increases with time, with optimum production at 3-4 weeks. Figure 7 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, Fig. 8 demonstrates a more rapid increase in the biosynthesis of ajmalicine (14) and yohimbine (15) (Corynanthé family) than observed for vindolinine (22) (Aspidosperma family). That is, the simple Corynanthé alkaloids ajmalicine (14) and yohimbine (15) reach maximum concentration at a much earlier period in culture growth than the biosynthetically more complex vindolinine (22). These are presumably derived from a common key intermediate, strictosidine (27), reflecting the differences in complexity of their biogenesis. Figure 8 also shows that at ca. 25 days, the concentration of these alkaloids begins to equilibrate, coincident with the onset of cell autolysis (Fig. 7). Figure 6 shows HPLC. traces of the later stages of growth period (3-7 weeks). Each sample contained ajmalicine (14) 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.

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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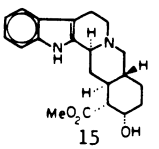
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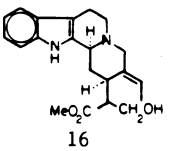
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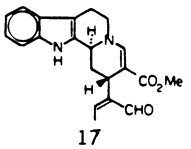
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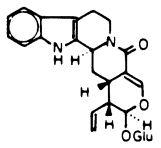
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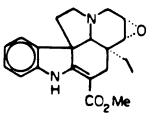
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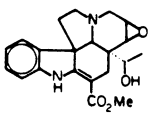
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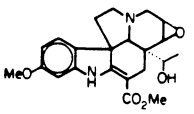
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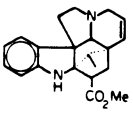
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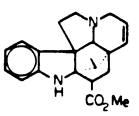
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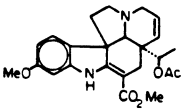
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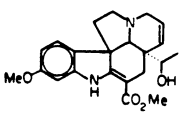
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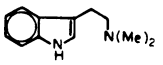
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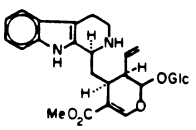
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The 200 GW line

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 953 line and produces its own "spectrum" of alkaloids as summarized in Table 3. Of particular interest is the alkaloid catharanthine (1, 0.005% dry cell wt) isolated for the first time in our studies. This line originally provided this alkaloid in amounts ca three times that normally obtainable from C. roseus plant material. Indeed, recent optimization studies with this line have shown even a further improvement.

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 (4) has been isolated[26-28]. Our initial studies with plant tissue cultures from the plant have not produced maytansine but several other cytotoxic agents have been isolated and characterized.

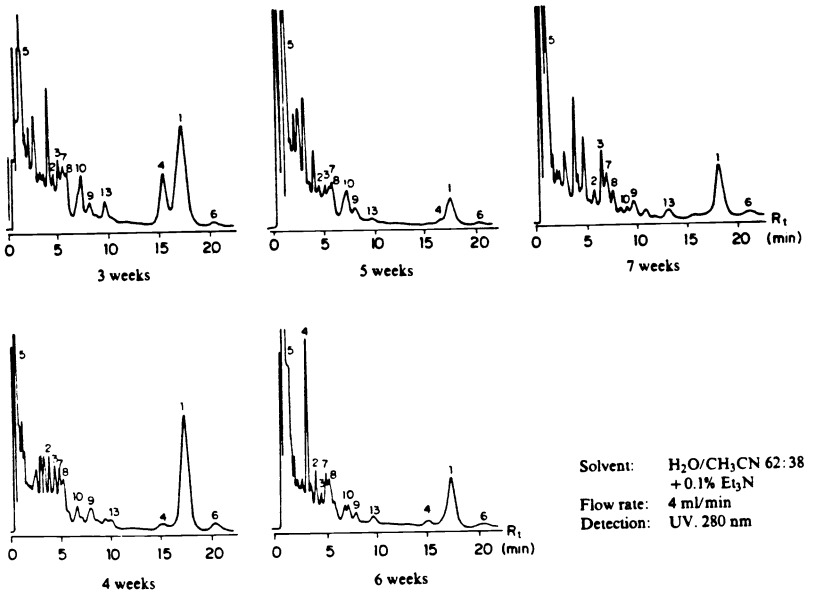

Fig. 6. Reverse phase HPLC. of alkaloid mixtures obtained after different growth periods.

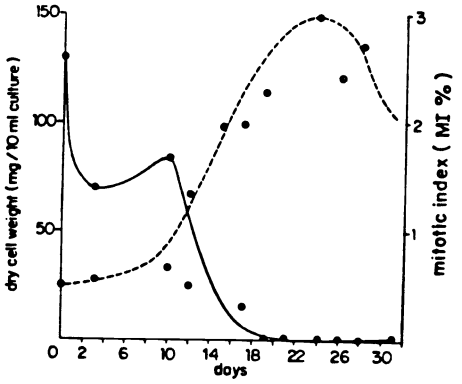

Fig. 7. Dry cell weight (---) and mitotic index (——).

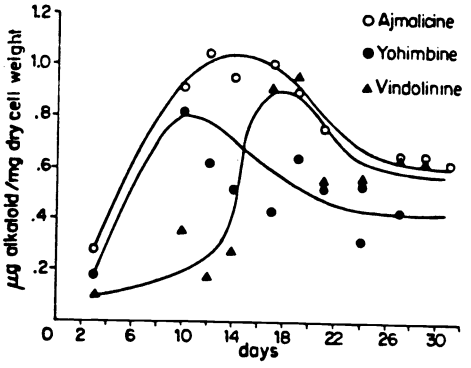

Fig. 8. Content of Ajmalicine (14), Yohimbine (15) and Vindolinine (22) at the earlier periods of culture growth.

Table 3. Alkaloids isolated from the 200GW Cell Line

Alkaloid	% Yield from dry cell wt.	% of crude alkaloid mixture
1	0.005	1.35
17	0.015	4.05
epimer of 17	0.026	7.02
14	0.006	1.62
20	0.002	0.54
21	0.005	1.44
22	0.002	0.54
23	0.002	0.54
18	0.224	60.48

% figures refer to isolated yields.

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, napthylacetic 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[29] showed some of the crude extracts to contain cytotoxic components ($ED_{50} < 5 \times 10^{0}$ ug/ml). This compares with an ED_{50} value of 2×10^{-1} ug/ml for crude plant extracts containing maytansine (see Table 4).

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 ug/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.

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 (28), tingenone (35) and 22β-hydroxytingenone (36) in yields of 0.043, 0.005 and 0.005% of dry weight of cells respectively. The acid (28) was characterised as its methyl ester (29) and identified by direct comparison with authentic samples. The two orange pigments tingenone and 22β-hydroxytingenone were identified by their mass and PMR spectra. Tingenone (35) 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} ug/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. Possible biosynthetic relationships between the above isolated compounds and other naturally occurring quinone-methides are summarized in Fig. 9. A detailed publication on these studies is available[30].

3) Studies with Tripterygium wilfordii

Tripdiolide (5, R = OH) and triptolide (5, 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)[31]. 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

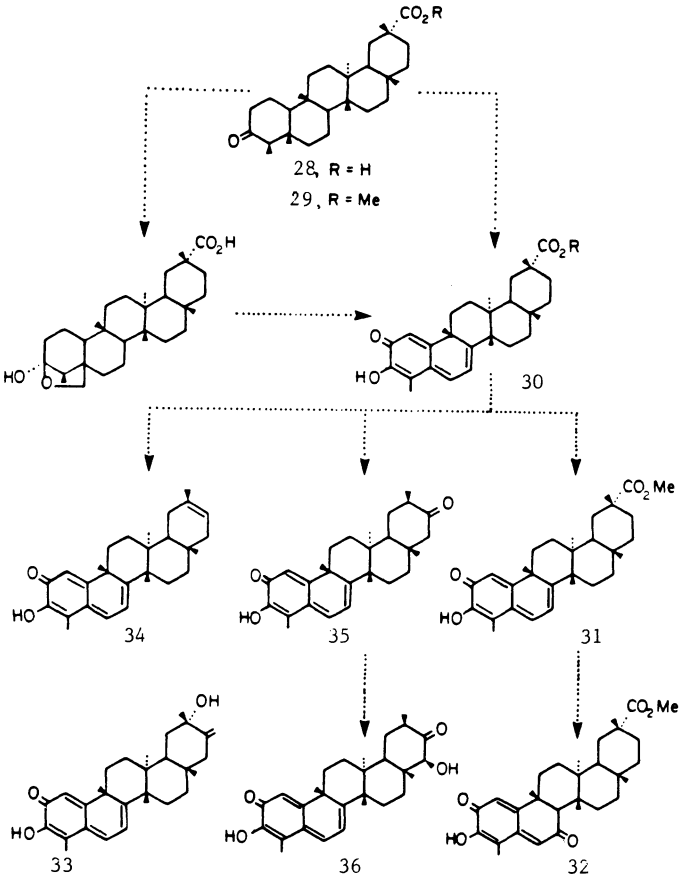

Fig. 9. Natural products isolated from M. buchananii tissue cultures and their biosynthetic relationships.

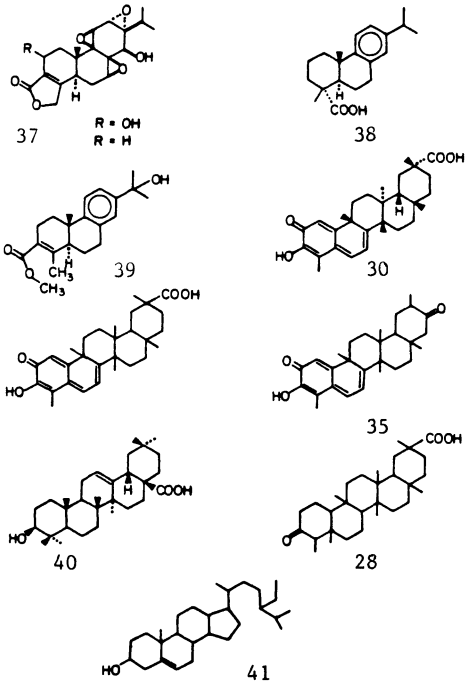

Fig. 10. Natural products isolated from T. wilfordii tissue cultures.

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 some of the established structures are summarized in Figure 10. Some of these are quinone methides similar to those encountered in the above studies with M. buchananii, while others are di- and triterpenoid in nature. At present, celastrol (30), polpunonic acid (28), oleanolic acid (40) and β-sitosterol (41) 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 recent optimization studies have provided levels 20 times that in the plant.

The isolation of dehydroabietic acid (38) and the unsaturated ester (39) provides interesting information about the possible biosynthetic pathway leading to the tripdiolide system (Fig. 11). It is suggested that 38 and the unsaturated carboxylic acid 42 are possible biosynthetic intermediates leading to tripdiolide. Appropriate experiments are presently underway.

Several publications concerning the above studies are available[32-34].

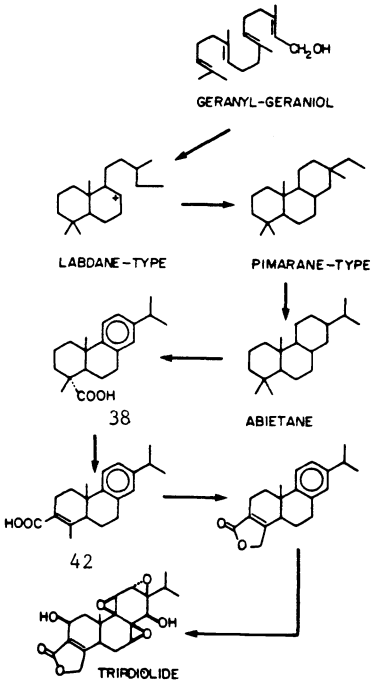

Fig. 11. Proposed biosynthetic pathway leading to tripdiolide.

4) Studies with cell free systems

Plant tissue cultures can provide excellent media for biosynthetic studies either directly with whole cells or with enzyme mixtures available from cell free systems. We have initiated some investigations with such systems in the hope of understanding the biosynthetic pathways involved with the above-mentioned natural products and, in particular, to attempt an evaluation of the enzymes responsible for optimum production of such target compounds. Experiments involving C. roseus will be presented here although similar methodology is being applied to T. wilfordii tissue cultures.

Brief mention has already been made (Figures 3 and 4) of earlier experiments[13-15] with cell free systems prepared from C. roseus leaves but a more detailed discussion is now appropriate in order to relate the results of the most recent investigations.

The purification procedure employed in all of the experiments concerned with C. roseus leaves and/or tissue cultures is summarized in Figure 12.

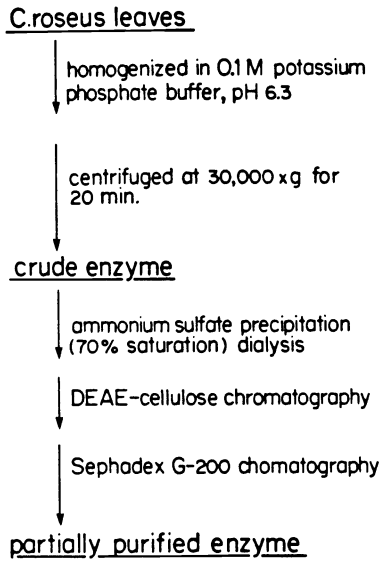

Fig. 12. General procedure for preparation of cell free systems from C. roseus leaves and/or tissue cultures.

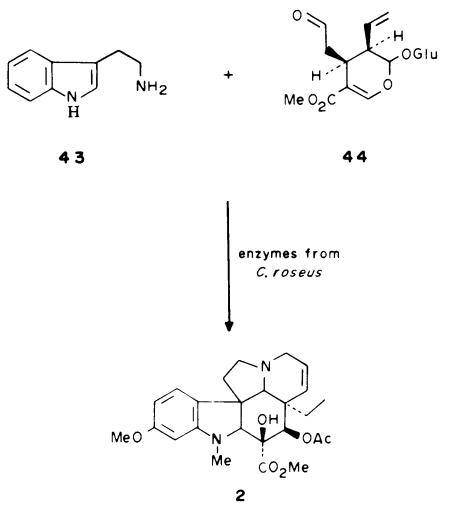

Fig. 13. The enzyme catalyzed synthesis of vindoline (2) from tryptamine (43) and secologanin (44).

The crude enzyme thus obtained was utilized in the experiments portrayed in Figs. 3 and 4 where important information concerning the late stages of the biosynthetic pathway of the bisindole alkaloids was obtained. In another series of experiments concerned with the biosynthesis of vindoline (2), we were able to demonstrate that this crude enzyme mixture was capable of transforming tryptamine (43) and secologanin (44) to 2 (Figure 13)[35].

Based on these earlier results, we proceeded to refine the methodology and obtain more information concerned with the enzymes involved in such conversions. Of particular interest to us was the enzyme(s) involved in the coupling of catharanthine (1) and vindoline (2) to the bisindole alkaloid (8) and its subsequent transformation to the other bisindole alkaloids (Figs. 3 and 4). Thus we have initiated a study directed at the recognition and purification of the relevant enzyme(s) involved in this coupling reaction.

The coupling enzyme activity was determined by monitoring the formation of 3',4'-dehydrovinblastine (8) and leurosine (11) using radiolabelled tracer techniques with (Ar-^{3}H) catharanthine and vindoline as substrates. We also applied HPLC methodology to analyse the protein contents of the cell free enzyme mixtures. The HPLC system employed two protein columns (Waters Associates I-250 and I-125) which were calibrated with a number of standard proteins. Table 5 lists the retention times for the respective protein under the conditions used for the analyses of the cell free extracts.

Figure 14 shows the HPLC profile of the crude enzyme from C. roseus leaves as prepared according to the procedure outlined in Fig. 12. It will be noted that a mixture of proteins varying in molecular weights of approximately 15,000 - 450,000 are present. In order to establish a relationship between the molecular size of the enzyme(s) involved in the coupling of catharanthine and vindoline to the bisindole system, we proceeded to further

Table 5. HPLC analysis of standard proteins (molecular weight versus retention time)

protein	molecular weight'	log (m.w)	retention time (min)
Ferritin	450,000	5.65	12.67
Catalase	240,000	5.38	14.07
Aldolase	158,000	5.20	14.60
Albumin (bovin serum)	68,000	4.83	14.66
Albumin (hen egg)	45,000	4.65	15.33
Horseradish peroxidase	40,000	4.60	16.51
DN ase	31,000	4.49	16.91
Chymotrypsinogen A	25,000	4.40	20.74
Cytochrome C	12,500	4.09	28.35

separate the cell free extract (crude enzyme) by precipitation, dialysis and chromatographic techniques to a "partially purified" enzyme stage (Figure 12).

The crude enzyme extract was brought to 70% saturation with ammonium sulfate. The precipitate thus formed was dialysed against phosphate buffer (pH 6.8) and the dialysate was applied on a DEAE-cellulose column equilibrated with potassium phosphate buffer (20 mM, pH 6.8). The elution profile of the DEAE-cellulose chromatography is shown in Figure 15.

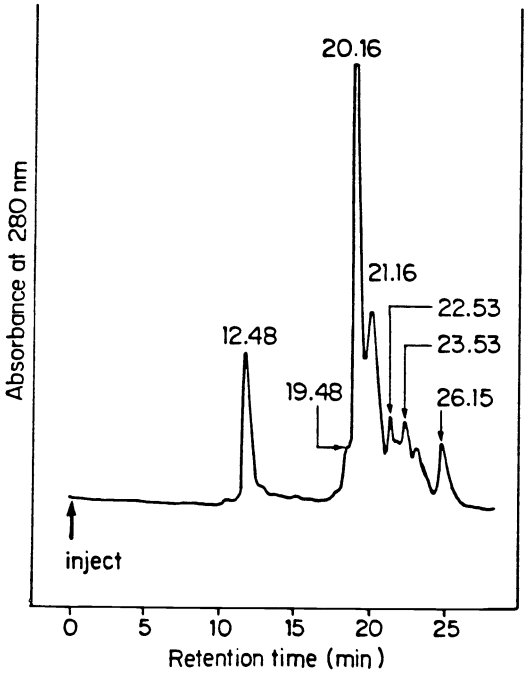

Fig. 14. HPLC analysis of cell free extract prepared from C. roseus leaves

Fractions 21 to 30 were found to possess the coupling enzyme activity and were therefore combined into Fraction II, and concentrated to a small volume by ultrafiltration. This concentrate was then subjected to Sephadex G-200 chromatography which exhibited two peaks as monitored by the uv absorbance at 280 nm (Figure 16). The fractions corresponding to the two peaks II-1 (fractions 9 to 22) and II-2 (fractions 23 - 29) were collected and analysed by HPLC (Figure 17) as well as assayed for coupling enzyme activity. Fraction II-1 which possessed the desired coupling enzymes activity was further fractionated by HPLC. Three fractions (A, B and C (see Figure 17)) corresponding to elution peaks of different retention times were collected and the results of their coupling enzyme activity determination are shown in Table 6.

It is clear from these investigations that the enzyme system(s) involved in the biosynthesis of 8 and 11 from the appropriate monomeric alkaloids are present in the short HPLC retention time region (11-20 min, Fraction A in Figure 17). From the calibration standards (Table 5) this indicates proteins of molecular weight greater than 25,000.

All of the above studies were performed with cell free systems obtained from C. roseus plants. We felt that a more preferable source would be tissue cultures in which a controlled and reproducible source of the enzymes could be available. Our most recent studies have taken this direction and some interesting results are already available. Crude enzyme extracts were prepared from various C. roseus tissue culture lines and HPLC analyses according to the above-mentioned method were performed. Figure 18 summarizes the results of four well-developed C. roseus lines from which various alkaloids have been isolated and characterized. The spectrum of alkaloids from the lines coded as "953" and "200GW" was discussed earlier. The "916" line is somewhat unique in that it exhibits normal growth characteristics but does not produce any of the alkaloids normally found in the other lines. A subline coded as "91601" and developed from "916" does produce alkaloids and the HPLC enzyme profiles, particularly in the region of 7-15 minutes retention time, are strikingly different. Such data are of considerable value in developing tissue culture lines with optimum production of target compounds and are also useful in biosynthetic investigations.

In summary, this lecture has provided some of the potentially important avenues in which we believe that plant tissue cultures will serve a valuable role. This aspect of biotechnology will continue to expand and provide interesting challenges not only to academic researchers but to those scientists involved in the commercial production of pharmaceutical agents.

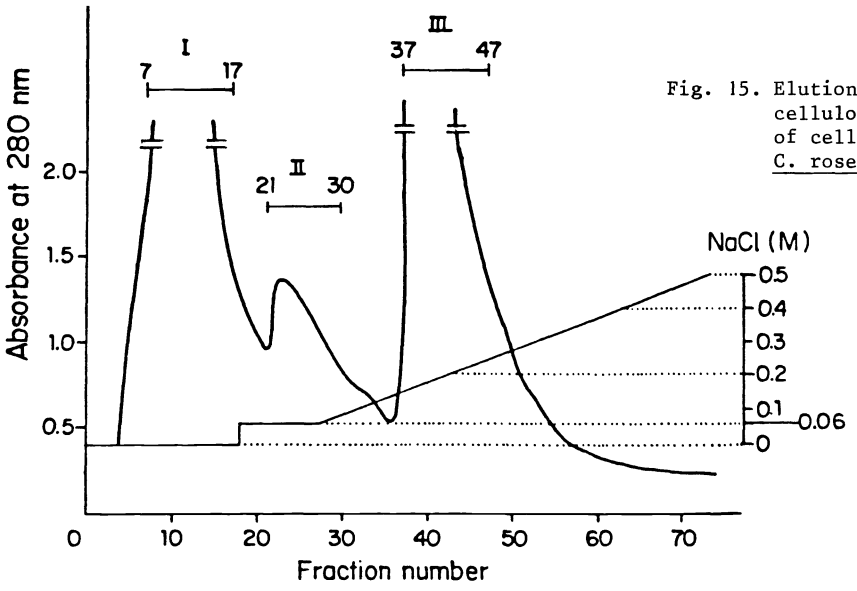

Fig. 15. Elution profile of DEAE cellulose chromatography of cell free extract from C. roseus leaves

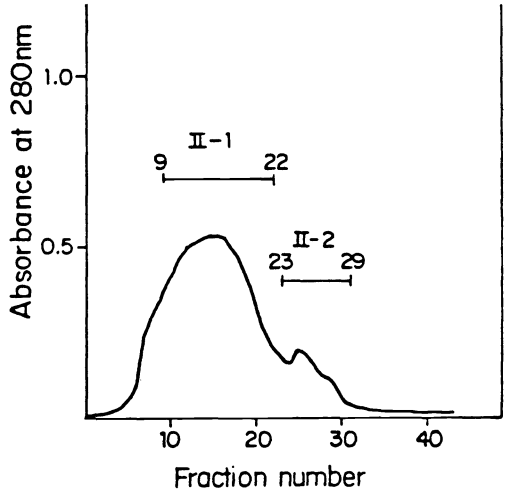

Fig. 16. Elution profile of Sephadex G-200 chromatography - Fraction II

Table 6. Preparative HPLC - Enzyme Fraction II-1 and evaluation of coupling enzyme activity

fraction	retention time (min)	net activity (dpm)	
		3',4-dehydro-vinblastine	leurosine
Frs. A	11 - 20	4137	3012
Frs. B	20 - 22.5	57	965
Frs. C	22.5 - 30	0	0

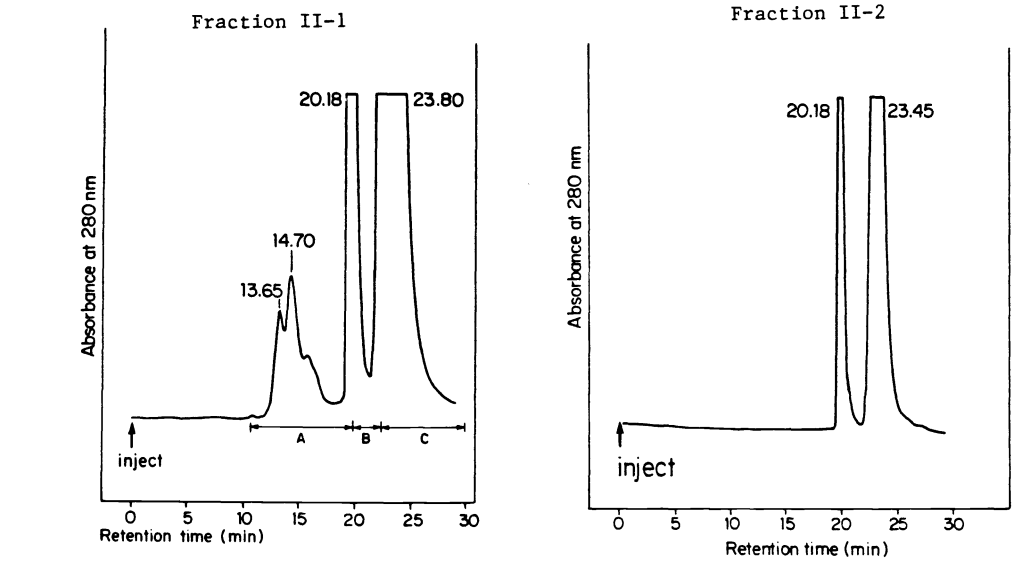

Fig. 17. HPLC profiles of fractions II-1 and II-2 from Sephadex G-200 chromatography

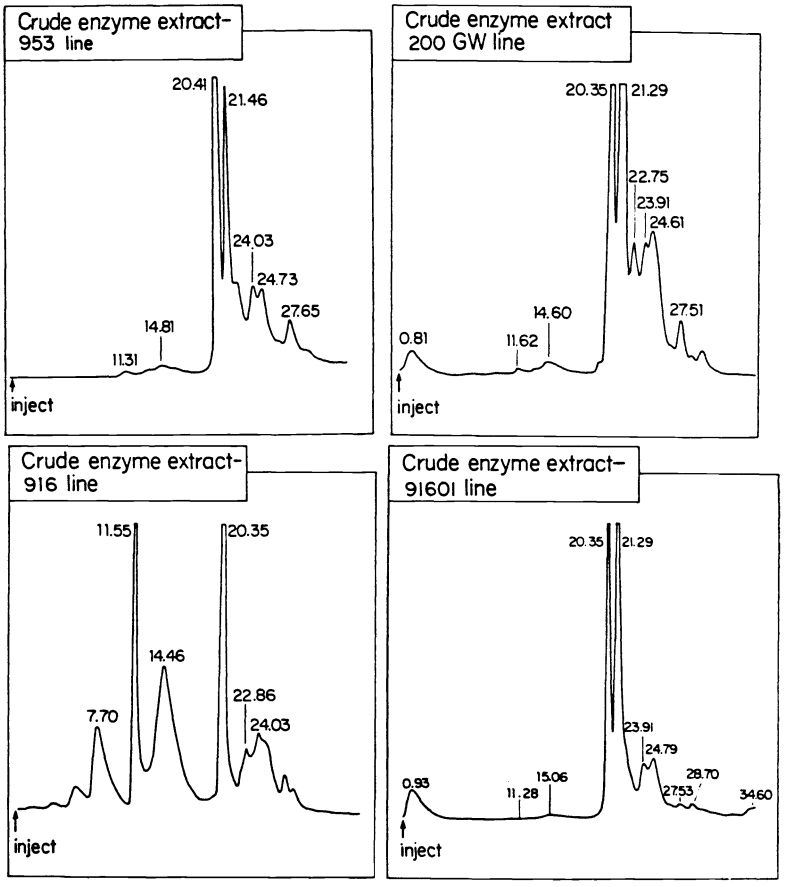

Fig. 18. HPLC profiles of crude enzyme extracts from several C. roseus tissue culture cell lines

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