STUDIES IN PLANT TISSUE CULTURE. THE SYNTHESIS AND BIOSYNTHESIS OF INDOLE ALKALOIDS*

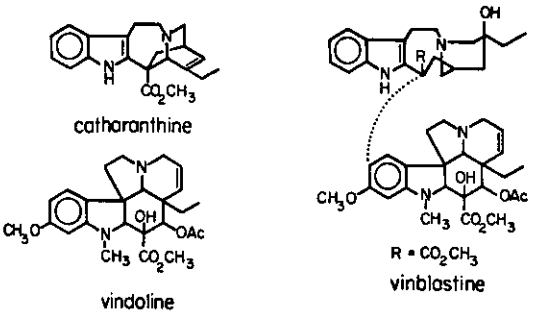
James P. Kutney
University of British Columbia, Department of Chemistry, 2036 Main Mall, Vancouver, British Columbia, Canada V6T 1Y6

Abstract - A summary of recent studies with various plant tissue cultures obtained from Catharanthus roseus is presented. The main emphasis of the research program concerns the propagation of stable cell lines for the production of the alkaloids catharanthine, vinblastine and vincristine. Related to these objectives are studied on the biosynthesis and biotransformation of 3',4'-anhydrovinblastine (26) in order to provide a better understanding of the enzymatic production of the above-mentioned bisindole alkaloids. Isolation of cell free extracts (crude enzyme mixtures) from such cultures and studies with these extracts are also presented.

The large family of indole alkaloids with its diversity in structure and biological activity has stimulated and maintained the interest of numerous investigators for many years. Studies concerning isolation and structure elucidation of plant derived alkaloids, their synthesis and biosynthesis have continued in recent years and it is highly likely that such investigations will be actively pursued into the future. A much more recent development within this area concerns the application of biotechnological methods, specifically plant tissue culture techniques, for the purpose of production of selected indole alkaloids, more detailed biosynthetic studies and investigations concerning enzyme related reactions.. Within this latter category, studies involving the alkaloids of Catharanthus roseus have received most attention. Our own program which was initiated approximately seven years ago has been directed toward several avenues within the Catharanthus area. These are: 1) development of stable cell lines for the production of the alkaloids catharanthine and vindoline; 2) development of

*This article is dedicated to Professor G. Stork on the occasion of his 65th birthday. The author expresses to Gilbert his sincere wishes for continued good health so that he may have the opportunity to pursue his excellent chemistry for a long time.

stable cell lines for the production of the clinical drugs vinblastine and vincristine (N-CH_3 in vinblastine replaced by N-CHO), either entirely by tissue culture methods or in combination with known synthetic chemistry methodology; 3) biosynthetic studies with such cell lines or with cell free extracts obtained from them; 4) enzyme reactions related to the aforementioned areas. Recent detailed articles [1,2] provide an overall summary of our earlier studies so this review will emphasize only those avenues under present investigation and which undoubtedly form the basis for future studies.



Alkaloid Production

Much data have been presented earlier about the nature and yields of alkaloids produced within a given cell line. It is clear that many factors (pH, nature of medium, age of culture, etc.) are responsible for producing a given spectrum of alkaloids and that the types and particularly yields of alkaloids can be varied by appropriate parameter changes during cell growth. Questions concerning reproducibility of such secondary metabolites by means of plant cell

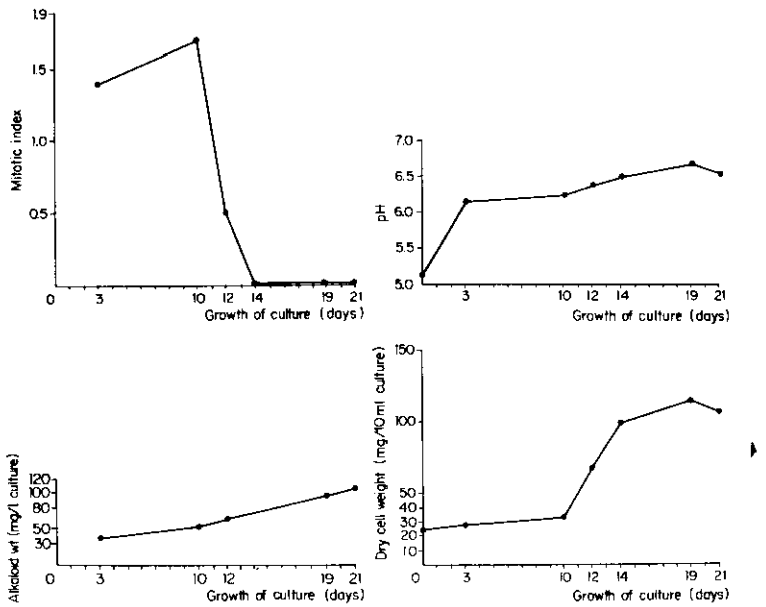


Fig. 1 General growth pattern of C. roseus tissue culture in bioreactor (cell Line 953).

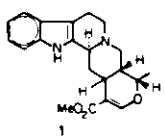
cultures have been raised by various authors but we have found that this is not a serious problem if appropriate parameters are controlled and carefully monitored. The general growth pattern of C. roseus tissue cultures in a bioreactor can be summarised as shown in Fig. 1. Thus measurement and monitoring of the parameters, pH, mitotic index, dry cell weight and alkaloid weight reveals important information about secondary metabolite production within a given cell line. Thus alkaloid production generally maximizes when the mitotic index reaches essentially zero, pH has attained a constant level and cell weight has maximised - all of these occurring in the 14-21 day period in this particular instance. Alkaloid levels can vary with time and culture method (shake flask versus bioreactor), as shown in Tables I and II for a specific cell line (953). Similarly, the spectrum of alkaloids produced, (structures 1 - 14), and their relative concentrations as monitored by HPLC (Fig. 2), varies with time.

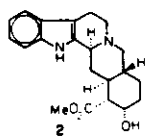
Table I. Alkaloid yields from batches of 953 line C. roseus cell cultures

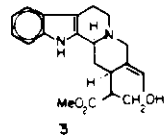
Sample	Culture method	Weight of freeze dried cells (g)	Weight of basic fraction (g)	% Alkaloid
1	Bioreactor (10 days)	90.5	0.168	0.185
2	Bioreactor (11 days)	110.0	0.178	0.16
3	Bioreactor (22 days)	26.9	0.058	0.21
4	Shake flask (14 days)	40.6	0.065	0.16
5	Shake flask (21 days)	49.66	0.182	0.37

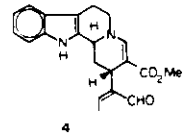
Table II. Alkaloid yields from 953 line C. roseus shake flask cultures

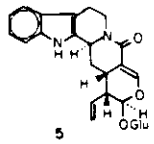
Cultivation time	Weight of freeze dried cells (g)	Weight of basic fraction (g)	% Alkaloid
3 Weeks	65.9	0.15	0.23
4 Weeks	51	0.15	0.29
5 Weeks	87.6	0.24	0.28
6 Weeks	19.8	0.125	0.63
7 Weeks	19.7	0.1	0.51

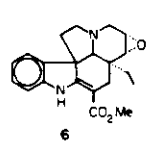


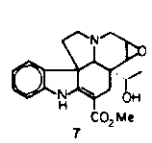


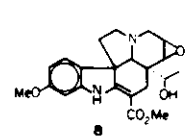


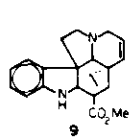


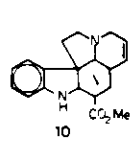


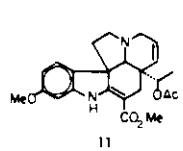


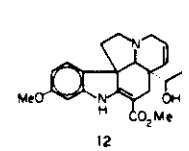


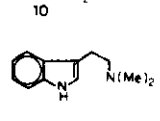


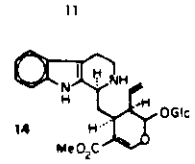












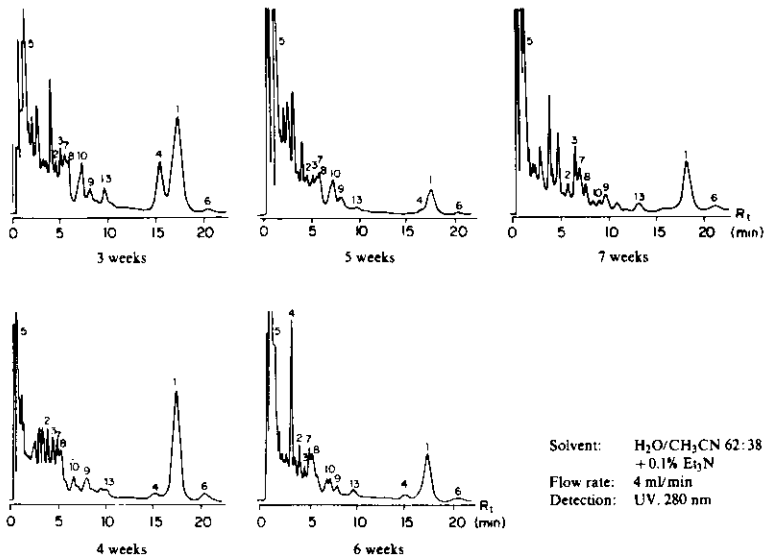


Fig. 2 HPLC monitoring of alkaloids produced in a bioreactor versus time (cell line 953).

Based on the data obtained from our earlier studies, as summarized thus far, we have initiated more recent experiments to evaluate the effect of bioregulators on the biosynthesis of the alkaloids ajmalicine (1) and catharanthine. For this purpose the previously developed 200 GW cell line [1,3], a good producer of these alkaloids, was selected.

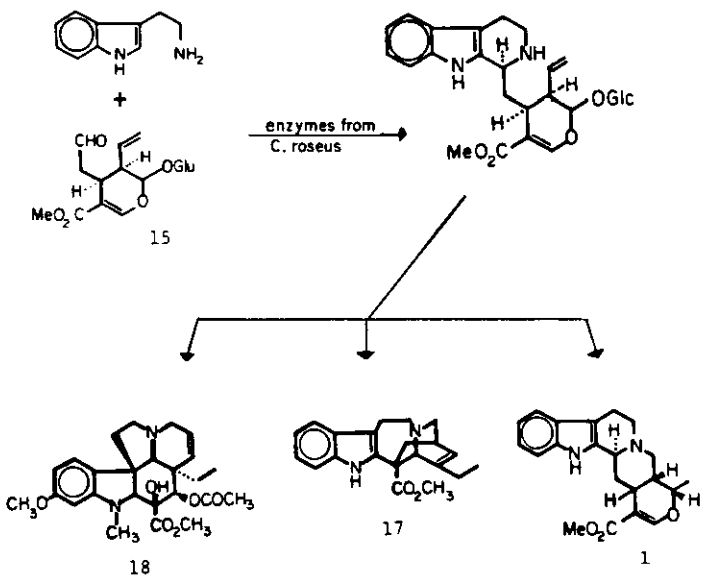
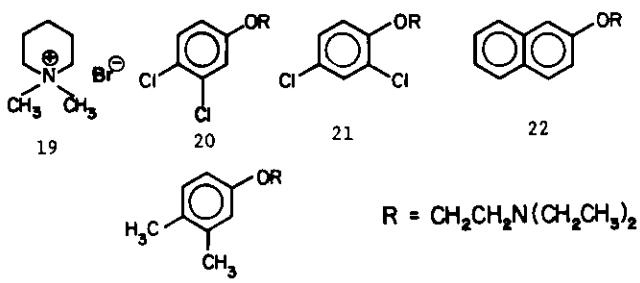


Fig. 3 Overall summary of the biosynthetic pathway of the alkaloids ajmalicine (1), catharanthine (17) and vindoline (18).

The rationale for the bioregulator experiments described below derives from the earlier studies of Yokohama et al.[4] who demonstrated that rubber biosynthesis is induced by certain aminoethyl phenyl ethers thereby implying that such synthetic bioregulators are stimulators of isoprenoid biosynthesis. Since secologanin (15) is an important isoprenoid precursor in indole alkaloid biosynthesis, it was logical to enquire as to whether such bioregulators would stimulate its production in C. roseus cell cultures. The well established biosynthetic pathway (Fig. 3) involving secologanin and tryptamine condensation to strictosidine (16), and the latter undergoing enzymatic transformation to ajmalicine (1), catharanthine (17) and vindoline (18) could then be invoked by the enzyme system of the tissue culture cell line to achieve the required biosynthesis of the target alkaloids.

The bioregulators chosen for this study are shown as structures 19-23.



19 20 21 22 23

In all our studies, the mitotic index remained at or near zero while the pH of the culture medium remained reasonably constant (pH = 5.0 - 5.6). No significant fluctuation of biomass in terms of cell dry weight was observed during an incubation period of 12-31 days when these bioregulators were added separately, at a concentration of 2 mg/l, to the cell cultures. Therefore, no inhibitory effect on cell growth is noted during this time course experiment (Fig. 4). In a second subsequent experiment, employing the two most promising bioregulators (19 and 23), cell biomass continued to increase for 6 to 8 days (Fig. 8) and then remained constant from day 12 as shown in Fig. 4.

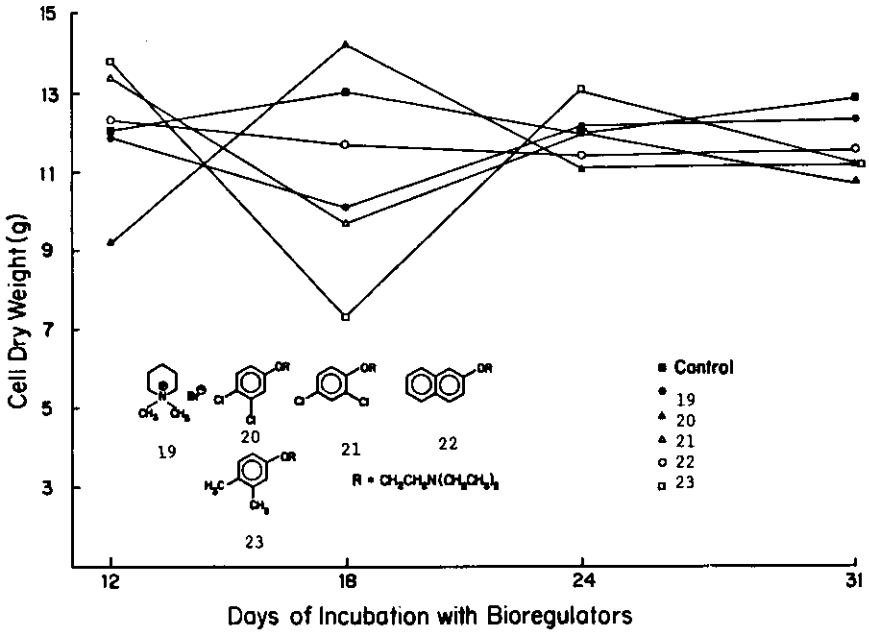


Fig. 4 Effect of bioregulators 19 to 23 on cell dry weight over an incubation period of 12 to 31 days.

There were significant differences in total alkaloid obtained (expressed as per cent of cell dry weight) with the different bioregulators and particularly after 12 and 18 days of incubation (Fig. 5). Of the five compounds evaluated, 19, 22 and 23 showed an increase while 20 and 21 showed a decrease in alkaloid production.

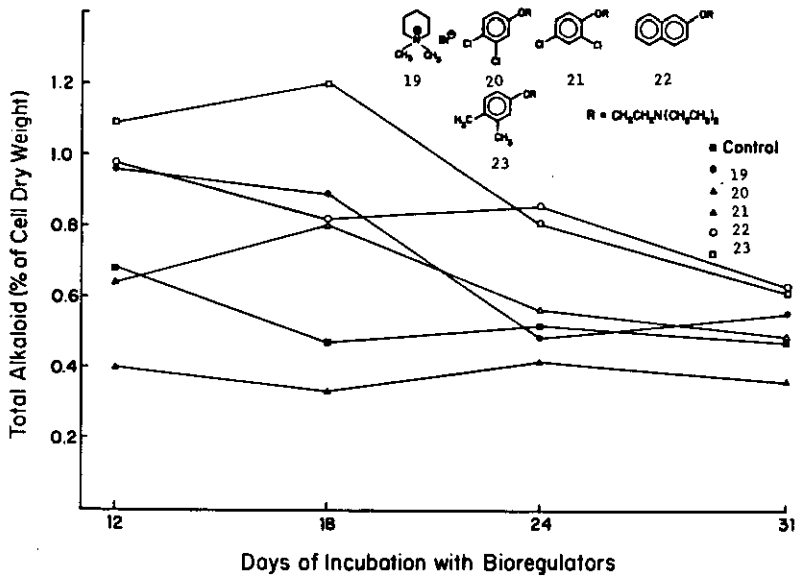


Fig. 5 Effect of bioregulators 19 to 23 on total alkaloid yield over an incubation period of 12 to 31 days.

Yields of the target alkaloids, ajmalicine and catharanthine, as monitored by HPLC (Fig. 6 and 7), indicate the different effects of the bioregulators on the biosynthesis of these compounds. In summary, the compounds 19 and 23 appeared to effect the best improvement particularly for catharanthine production and were therefore evaluated in a second time course experiment (Figs. 8 - 11).

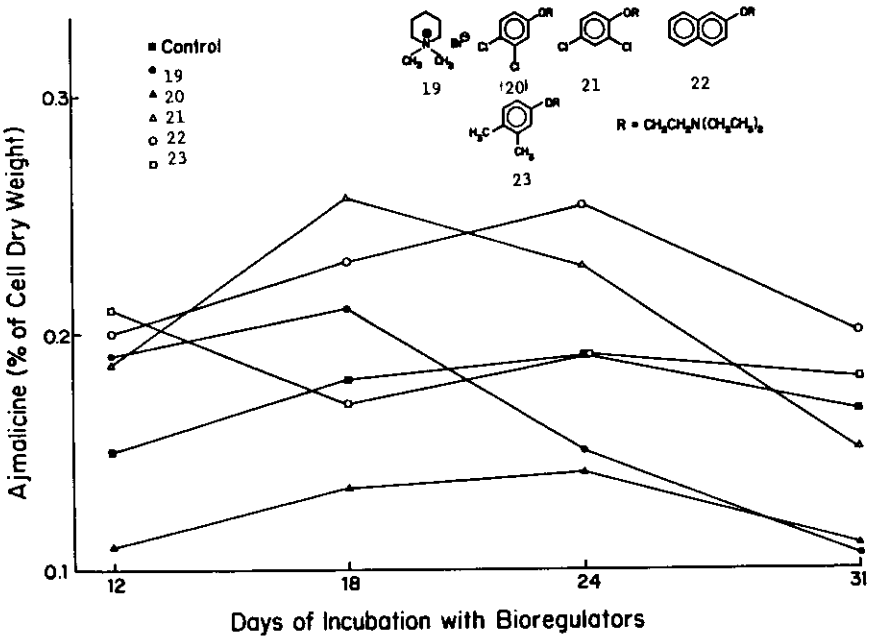


Fig. 6 Effect of bioregulators 19 to 23 on yield of ajmalicine over an incubation period of 12 to 31 days.

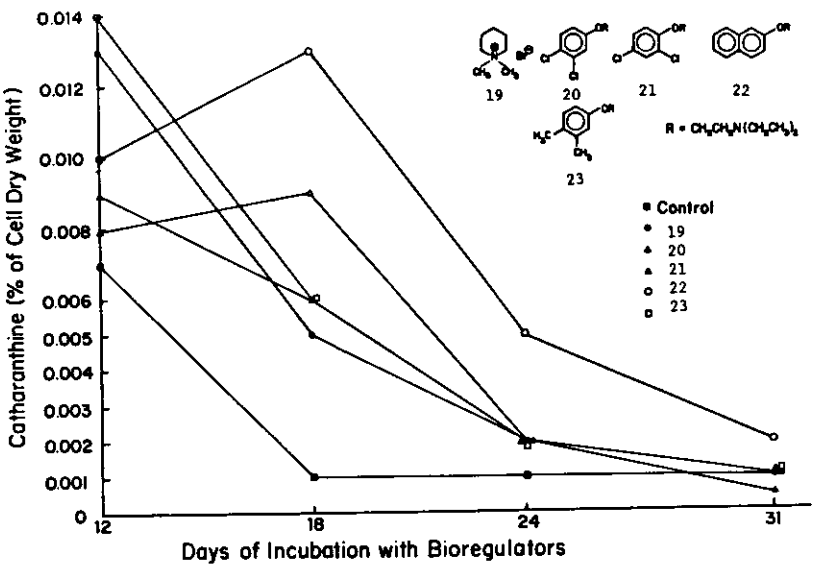


Fig. 7 Effect of bioregulators 19 to 23 on yield of catharanthine over an incubation period of 12 to 31 days.

In the second experiment with the 200 GW cell line, the bioregulators 19 and 23 were incubated for shorter time periods (4,6,8 and 10 days). Fig. 8 illustrates the increase in biomass over a 4 - 10 day period.

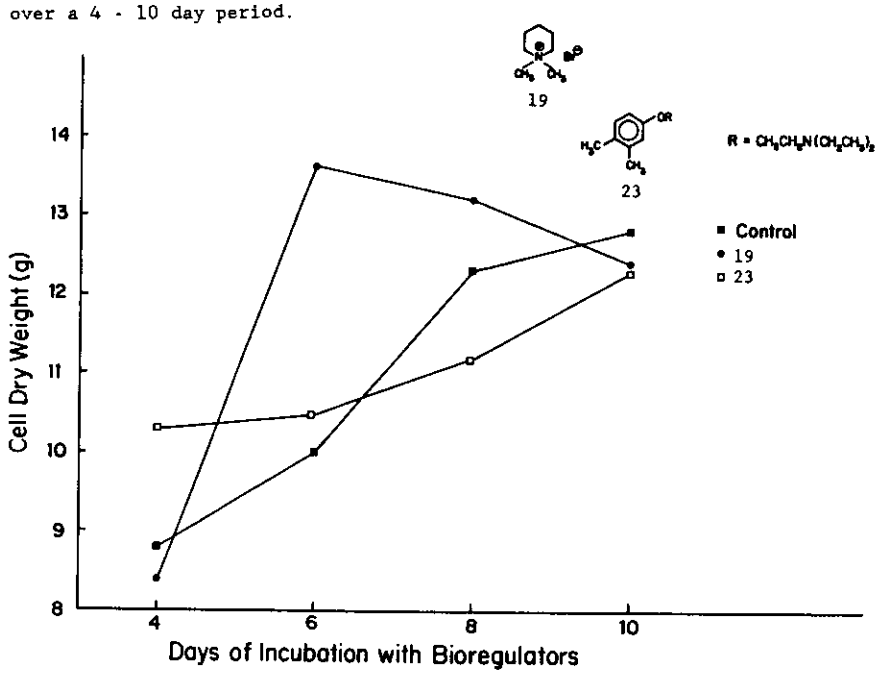


Fig. 8 Effect of bioregulators 19 and 23 on cell dry weight over an incubation period of 4 to 10 days.

Total alkaloid production (Fig. 9) reached a maximum level after 6 days of incubation with the highest yield being obtained with 23. Bioregulator 19 also produced more than the control sample.

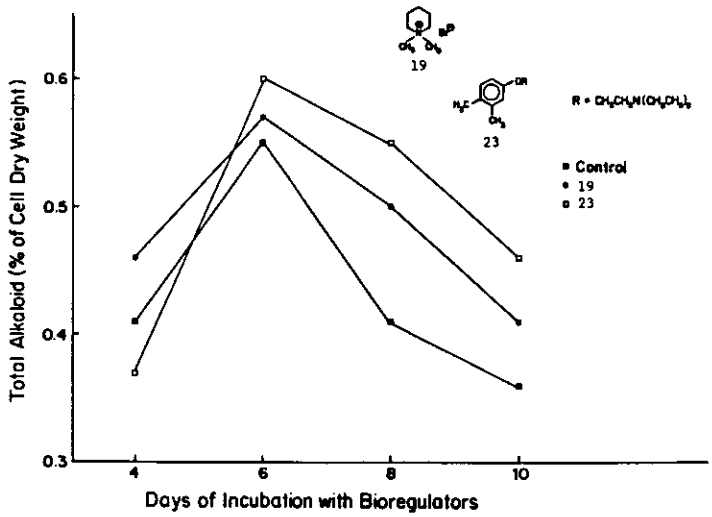


Fig. 9 Effect of bioregulators 19 ans 23 on total alkaloid yield over an incubatin period of 4 to 10 days.

Figs. 10 and 11 illustrate the significant increases in ajmalicine and catharanthine production when the bioregulators 19 and 23 are employed.

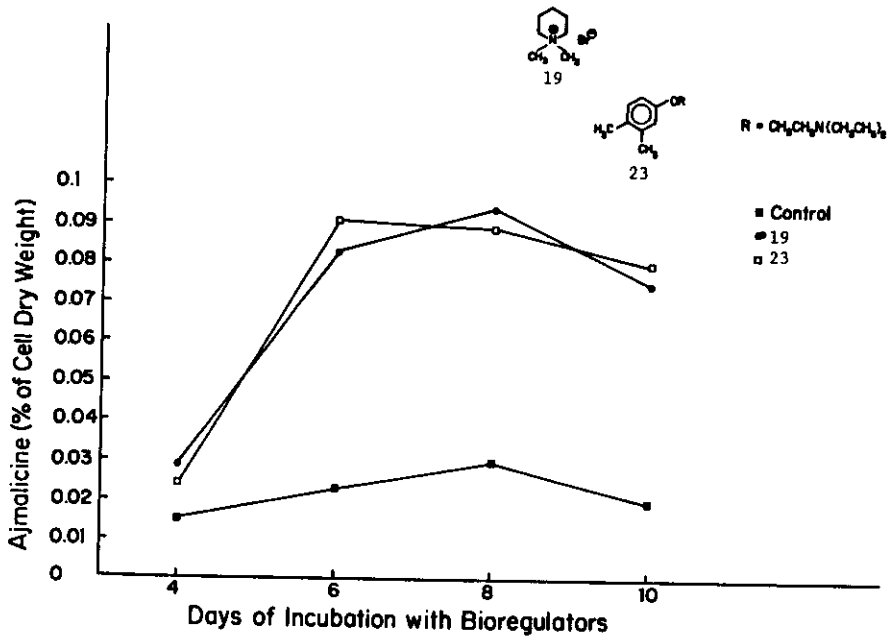


Fig. 10 Effect of bioregulators 19 and 23 on yield of ajmalicine over an incubation period of 4 to 10 days.

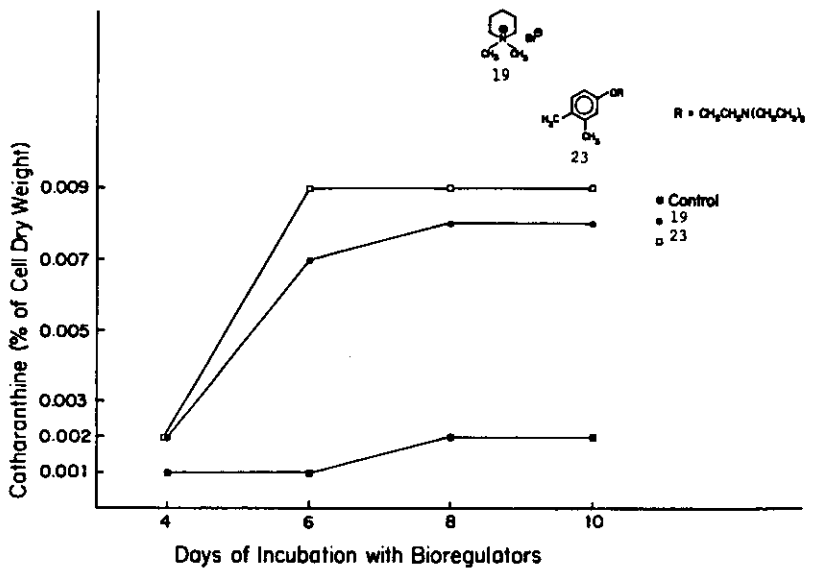


Fig. 11 Effect of bioregulators 19 and 23 on yield of catharanthine over an incubation of 4 to 10 days.

It should be noted that a similar but independent study by Scott et al[5], has revealed that the 2,4-dichlorophenyl ether derivative (21) was most effective in promoting ajmalicine and catharanthine production in their cell line. Such differences between the results of the two laboratories suggest that different cell lines produced in various laboratories may indeed reveal different patterns of bioregulator stimulation of alkaloid production. Our studies on the effects of bioregulators have recently appeared in print[6].

Biosynthesis and Biotransformation Studies -
Comparisons of Enzymatic Reactions with Laboratory Syntheses

Alkaloid production in tissue culture employing various nutrient media, to which appropriate additives are made, as noted above, can afford interesting and encouraging results. Alkaloid levels significantly higher than those normally found in the living plant can be achieved but the time of production (often 1 - several weeks) may preclude, at least in some instances, the use of this methodology for commercial production of appropriate pharmaceuticals etc. On the other hand, the use of plant enzyme systems, conveniently generated via tissue culture techniques, can afford a powerful alternative to the well established and highly important methods of biotransformation employing fungal and/or bacterial strains of micro-organisms. It is known from the structural analyses of plant-derived natural products that plants undoubtedly contain enzyme systems which are not necessarily present in micro-organisms and their use for biological transformation of organic substrates, particularly in instances where transformations by microbial strains have failed, is of considerable interest. In effect, such biotransformations may be considered in a complementary sense to direct production of the target compound by tissue culture methods, as discussed above. Indeed such considerations for production of Catharanthus alkaloids were pursued in our program and have provided important information. An understanding of the possible modes of enzyme-catalyzed biotransformations brings into consideration, in simultaneous fashion, an understanding of biosynthetic pathways involved. In effect, experiments designed to achieve appropriate biotransformations shed light on the biosynthesis of the target compounds.

In our earlier studies[7] concerned with chemical syntheses of the bisindole alkaloids, the coupling of catharanthine, via its N-oxide intermediate (24), with vindoline (18) in the presence of trifluoroacetic anhydride and subsequent reduction of the resulting iminium intermediate (25) with sodium borohydride, afforded the desired 3',4'-anhydrovinblastine (26) system (Fig. 12).

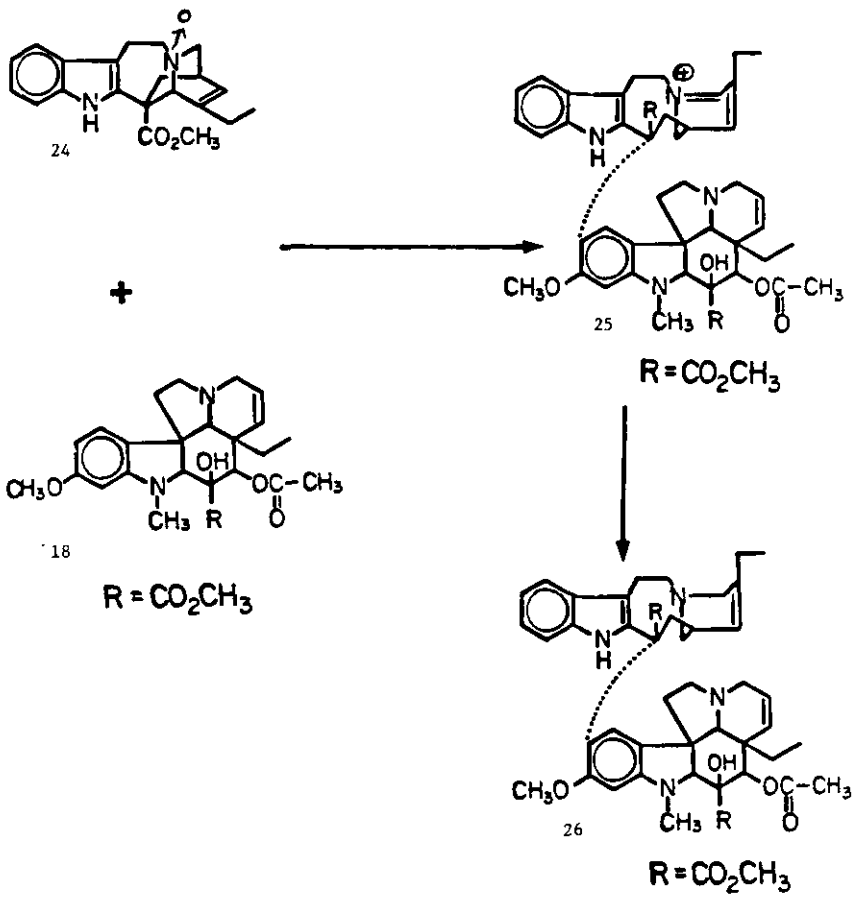


Fig. 12 Synthesis of 3',4'-anhydrovinblastine (26) via chemical coupling of catharanthine N-oxide (24) with vindoline (18).

A great deal of effort was then expended in our laboratories in order to elaborate 26 into the natural alkaloids, leurosine (27), catharine (28), vinblastine (31, R=CH_3), vinamidine (29, R=H) etc. Indeed, in spite of the structural complexity of 26, it was possible to selectively functionalise this system so as to complete syntheses of a number of these alkaloids. Figs. 13 and 14 summarise our results in this area.

Fig. 13 illustrates the chemical interconversions [8,9] of 26 with oxygen and tert-butyl hydroperoxide. Thus oxidative transformation of 26 affords leurosine (27), which on further oxidation yields catharine (28).

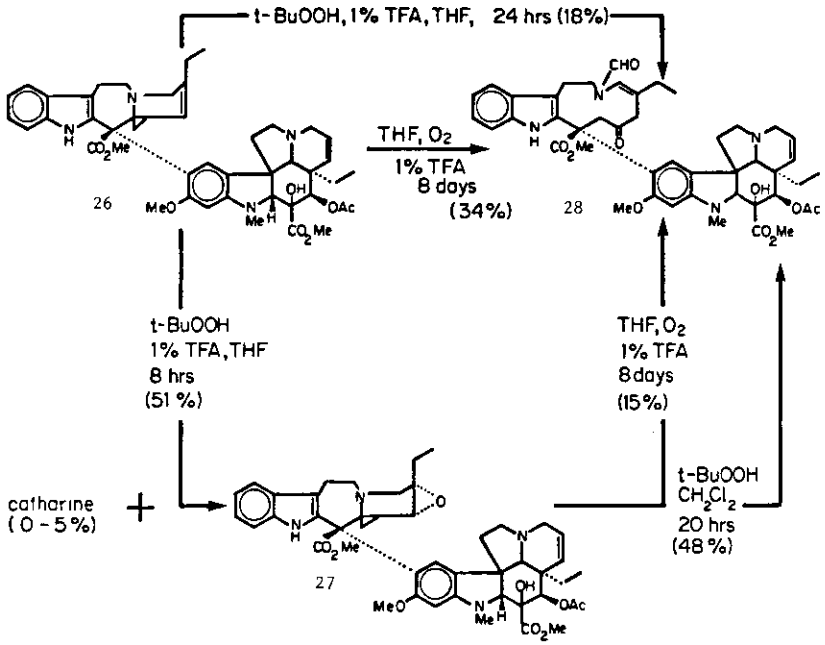


Fig. 13 Synthesis of leurosine (27) and catharine (28) from 3',4'-anhydrovinblastine (26)

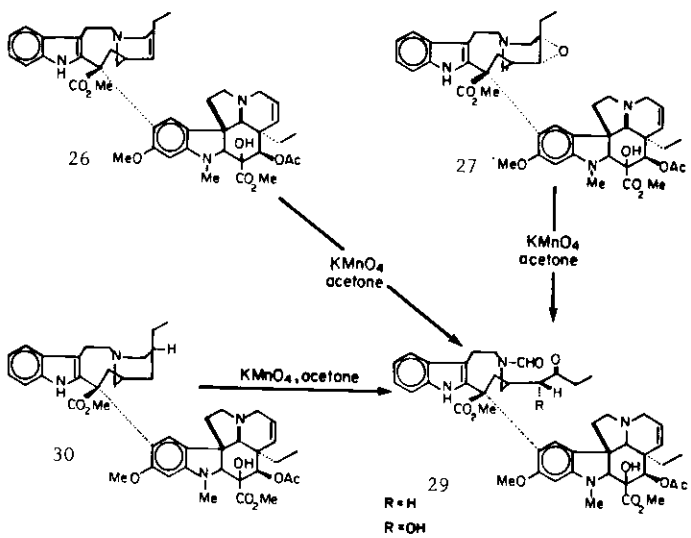
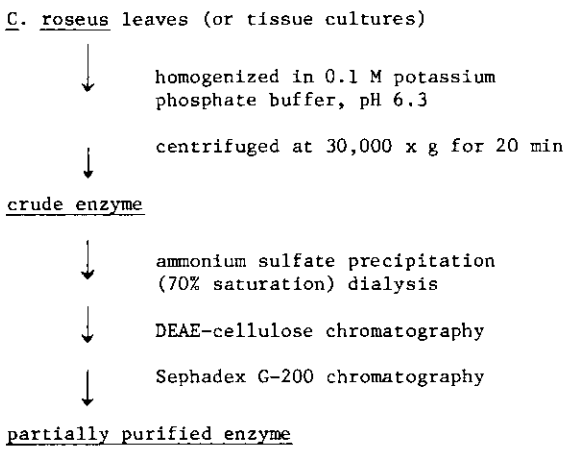


Fig. 14 Synthesis of vinamidine (29,R=H) and 3R-hydroxyvinamidine (29,R=OH) - overall summary

Fig. 14 summarises our detailed investigations[9] in which potassium permanganate is employed as the selective oxidising agent. The substrate, 3',4'-anhydrovinblastine (26) converts to hydroxyvinamidine (29, R=OH) as does leurosine (27). On the other hand, 4'-deoxyleurosidine (30), the catalytic reduction product of 26, transforms to the natural alkaloid vinamidine (29, R=H).

As will be seen later, these selective reactions in the chemical laboratory, completed before any studies with plant tissue cultures were initiated, have proven to be of considerable importance in the present program since there is remarkable similarity of the earlier results with those presently obtained with cell cultures and/or cell free extracts obtained from such cultures.

Scheme 1



The chemical coupling of catharanthine, via an oxidative process, with the dihydroindole system of vindoline, raised the question as to whether a similar coupling may not be occurring within the enzyme system of the plant. For this purpose, a preparation of a cell free extract (crude enzyme in Scheme 1) from C. roseus live plants had to be developed. Scheme 1 outlines the method employed in our initial studies[10] in this direction.

Employing tritium-labelled catharanthine and carbon-14 labelled vindoline, it could be shown that the enzymic system present in the cell free extract, as obtained from C. roseus leaves according to the procedure of Scheme 1, does indeed perform the coupling reaction. In shorter incubation periods, the major product isolated is 3',4'-anhydrovinblastine (26) but longer incubation times reveal leurosine as the main product. Fig. 15 summarizes the results obtained. Similar results were obtained in an independent study by Scott[11].

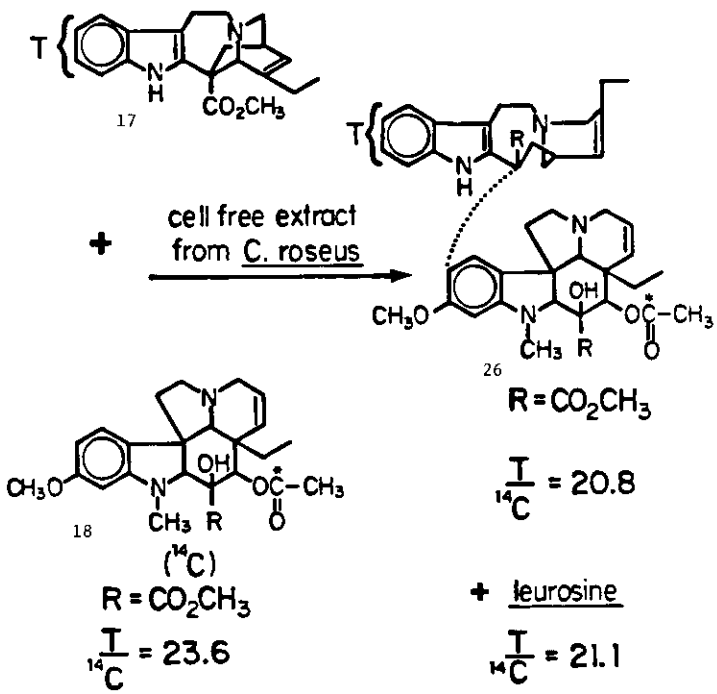


Fig. 15 Biosynthesis of 3',4'-anhydrovinblastine (26) and leurosine employing cell free extracts from C. roseus leaves.

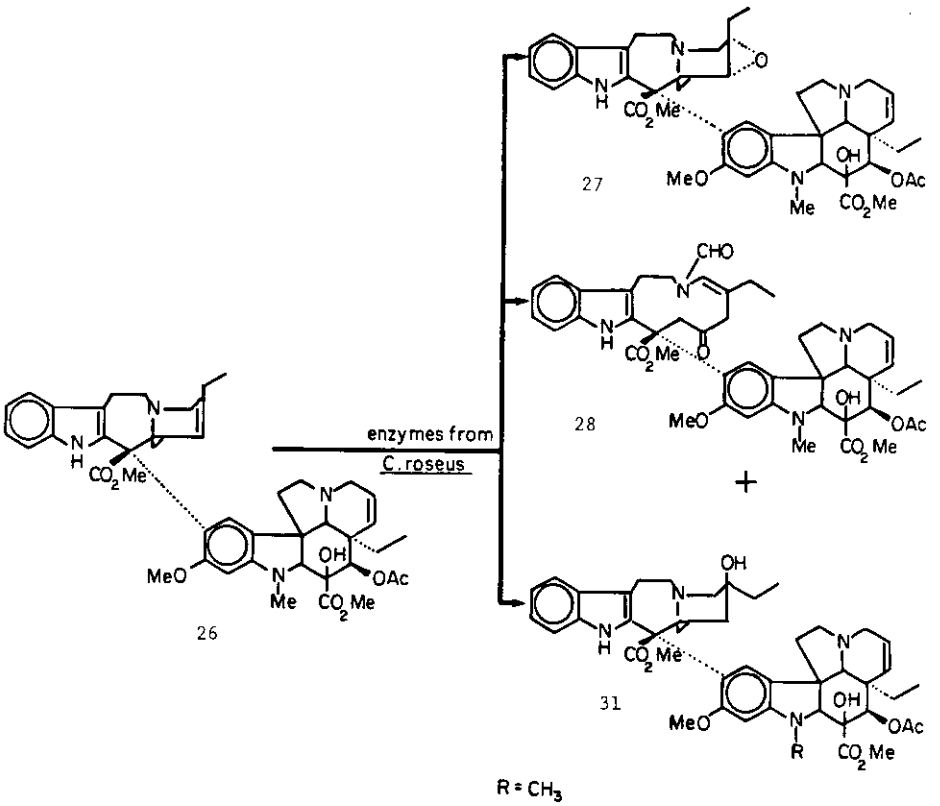


Fig. 16 Enzyme catalyzed synthesis of leurosine (27), catharine(28), and vinblastine (31), employing cell free extracts from C. roseus leaves.

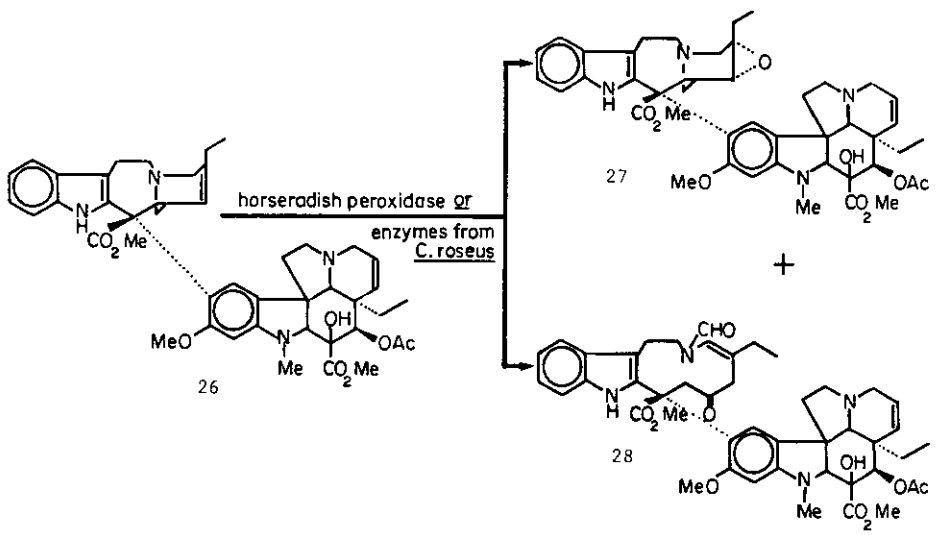


Fig. 17 Enzyme catalyzed synthesis of leurosine (27) and catharine (28) - comparison of horseradish peroxidase and cell free extracts from C. roseus leaves.

The enzymatic conversion of 3',4'-anhydrovinblastine (26) to leurosine (27), catharine (28) and the important clinical drug vinblastine (31, R=CH_3) was also established in subsequent experiments[10] and Fig. 16 summarises the data. Vincristine was not found in this bioconversion.

The earlier chemical conversion of 26 to leurosine and catharine by means of tert-butyl hydroperoxide (Fig. 13) raised the possibility that the enzymes responsible for the conversion of 26 to leurosine and catharine and present in the isolated cell free extract, were "peroxidases" and were performing an enzymatic function similar to that observed in the chemical laboratory. Assay of the cell free extract by methods normally employed for commercial horseradish peroxidase indeed revealed the presence of such enzymes. Furthermore, an experiment involving commercial horseradish peroxidase and run in parallel with the cell free extract biotransformation, revealed that the commercial enzyme behaved similarly in transforming 26 to the alkaloids leurosine and catharine (Fig. 17).

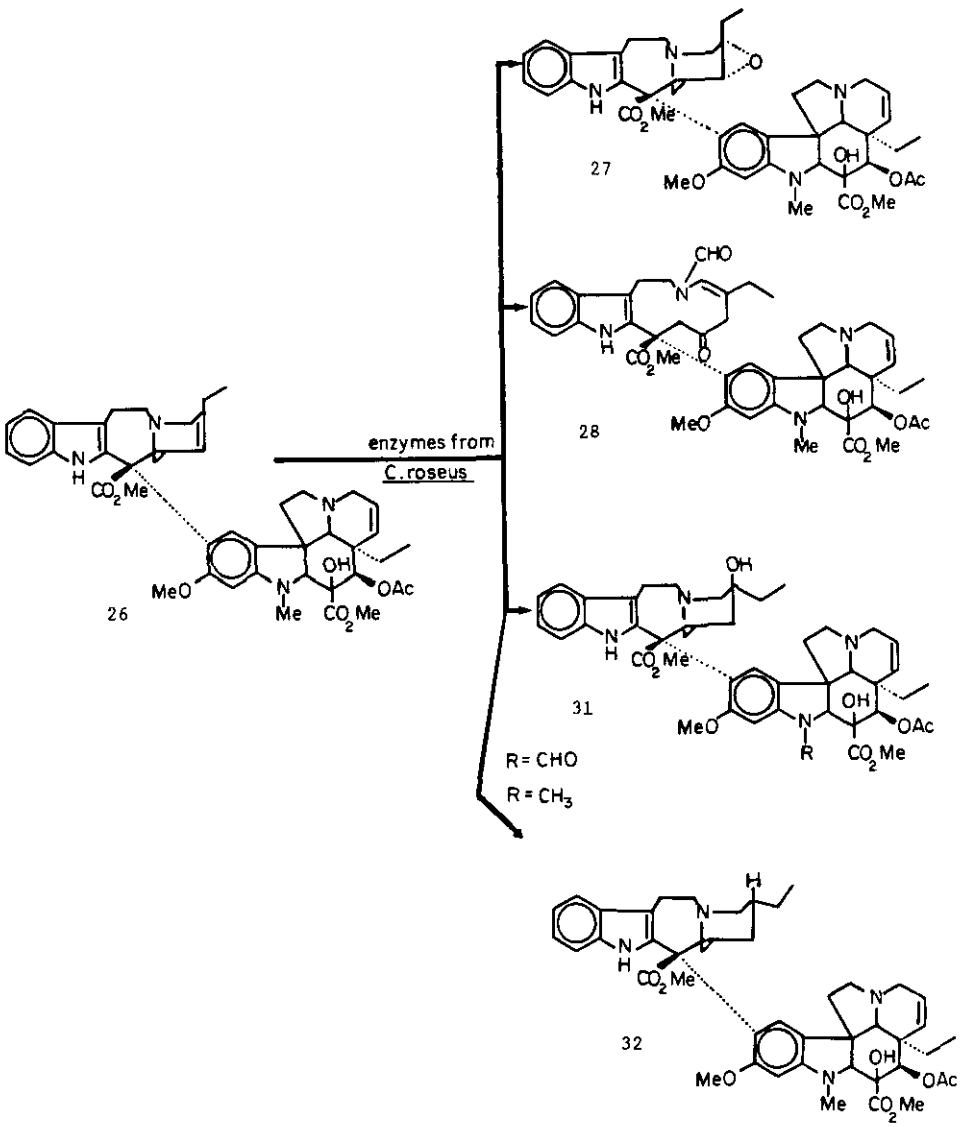
With the above information in hand, our attention turned to studies with the already established stable tissue culture cell lines of C. roseus since it was clear that such cell lines would provide a reliable source of enzyme systems for achieving reproducible results relating to the biosynthesis, biotransformation and production of the bisindole alkaloids.

Our initial studies[12] concerned the biotransformation of 26 and employed whole cells of the 916 cell line - a unique line in that it exhibited satisfactory growth characteristics but did not produce any alkaloids. Incubation of 26, as the hydrosulfate salt for varying time periods (2 - 72 h), revealed that the best results were obtained with 24 - 48 h incubations. Under the latter conditions the alkaloids leurosine (25 - 30% yield) and catharine (9-16% yield) were obtained with about 40% of 26 being utilized by the cells. Optimum conditions for the biotransformation of 26 were not developed so it is clear that higher yields of the end products could be achieved.

Since neither vinblastine nor vincristine were obtained in the above experiments with the 916 cell line, experiments concerning the biotransformation of 26 were conducted with alkaloid producing lines, for example, the previously developed 200 GW line [3] and lines developed recently in our laboratories.

Experiments with "growing" cells of the 200 GW line involved incubation times of 15 - 150 h. After 150 h incubation, no substrate remained but the incubation time was clearly too long and identification of any metabolites proved difficult due to the complexity of the product mixture. The alkaloid catharine was identified as a minor component.

Fig. 18 Summary of biotransformation of 3',4'-anhydrovinblastine (26) with "Growing" Cells of 200 GW/Cell line.



Shorter time periods (15 - 30 h) showed a less complex mixture and revealed very interesting results. Thus, a 30 h incubation period revealed the alkaloids leurosine (27) and catharine (28) while the 15 h incubation revealed the presence of these two alkaloids and, for the first time, the presence of the clinically important drugs vinblastine (31, R=CH_3) and vincristine (31, R=CHO) and 4'-deoxyleurosidine (32). Fig. 18 summarizes these results.

Utilizing cell lines recently developed in our laboratories, it was possible to obtain additional important information about the biotransformation of 3',4'-anhydrovinblastine (26) and the corresponding fate of the metabolites formed. These studies have involved experiments with growing cells as well as cell free extracts prepared according to the procedure of Scheme 1. Fig. 19 summarizes the results of a large number of experiments and pertinent comments concerning the most important features are presented below.

Employing an incubation period of 15 h, and growing cells of our recently developed lines, the following additional information has been obtained. Additional metabolites of 3',4'-anhydrovinblastine have been identified and these are vinamidine (29,R=H) and hydroxyvinamidine (29, R=OH). Other bisindole products are also present but are not as yet identified.

The important question as to whether the enzymes responsible for these biotransformations are present within the cells and remain there during the entire growth period (5 - 23 days in the nutrient medium) was answered in the following manner. Filtration of the cell material after a given growth period provided the "spent" medium which was simultaneously evaluated with the cell free extract obtained from the cells. Enzymatic activity in the "spent" medium is minimal, in terms of biotransformation of 26, when a short growth period, for example, 5 days, is involved. On the other hand, older cultures, for example, 19 - 23 days old, which reveal significant cell lysis do provide a "spent" medium with significant enzymatic activity. These results indicate that the enzymes involved are maintained within the cell and are released into the medium if significant cell lysis occurs. For example, an experiment involving "spent" medium from a 17 day old culture and 26 as substrate in a 15 h incubation period, revealed the presence of leurosine (27), catharine (28), vinamidine (29, R=H) and hydroxyvinamidine (29, R=OH), the latter as a major component. Vinblastine was not detected.

The cell free extract obtained from the 17 day old culture, as noted above, was evaluated simultaneously as to its enzymatic activity in terms of biotransformation of 3',4'-anhydrovinblastine. After only a short incubation period of 3.5 h, the important metabolite,

vinblastine was noted, in addition to the other previously identified metabolites. Hydroxyvinamidine (29, R=OH), a major metabolite in the "spent" medium experiment is now shown only as a minor component.

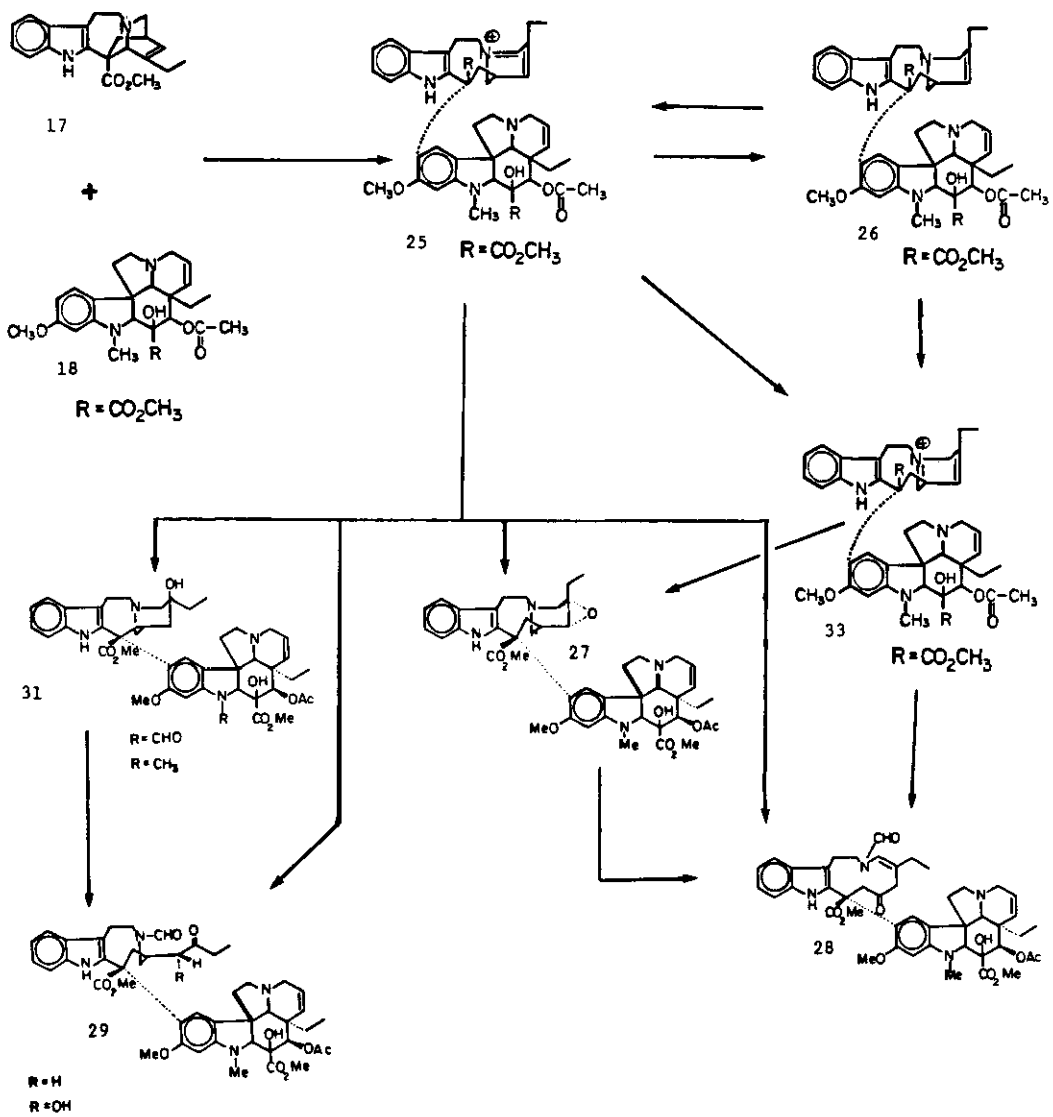


Fig. 19 Overall summary of results obtained when catharanthine (17), vindoline (18) and 3',4'-anhydrovinblastine (26) are incubated either with cell cultures of Catharanthus roseus or cell free extracts derived from such cultures.

The differences in the results obtained from the above two experiments may be rationalized in several ways since the incubation periods are different (15 h versus 3.5 h). On the one hand, it could be argued that the enzyme(s) system responsible for "hydration" of the 3',4'-double bond in 26 to yield vinblastine is not released into the "spent" medium or alternatively, the longer incubation period has allowed enzymatic conversion of vinblastine to other metabolites. In fact, in a separate study, vinblastine (31, R = CH_3) when incubated in a cell free extract, under similar conditions, is metabolized rapidly (HPLC monitoring) and is completely converted within 3-5 h. The main products observed are vinamidine and N-formylvinamidine (structure 29, R=H, N-CH_3 replaced by N-CHO). In a parallel study, vincristine (31, R = CHO) is similarly metabolized to N-formylvinamidine in this time period (see Fig. 19, 31 --> 29). In summary, it can be stated that the natural product vinamidine, its hydroxy derivative and the corresponding N-formyl analogues represent the ultimate end products of enzymatic oxidation.

Further additional information bearing on the possible mechanistic pathway concerning the biotransformation of 26 to the above metabolites was also obtained from our cell free extract experiments. It is clear that iminium intermediates such as 25 and 33 (Fig. 19) are products of enzymatic oxidation of 26 and their conversions to the various metabolites, leurosine (27), catharine (28), vinblastine (31, R=CH_3), vincristine (31, R=CHO), vinamidine (29, R=H) and hydroxyvinamidine (29, R=OH), as suggested in Fig. 19, can be expressed via reasonable mechanistic pathways. For this reason, it was considered important to detect the formation of such iminium species in the incubation mixture. Indeed this has now been accomplished.

Employing HPLC (reverse phase, C_{18} column), the biotransformation of 26 can be monitored over various time periods with the specific objectives of detecting 25 and/or 33. In order to establish the appropriate retention times for such iminium intermediates, an authentic sample was prepared via the catharanthine -- vindoline coupling reaction (Fig. 12) in which 25 is unambiguously formed. With cell free extracts and 26 as substrate, an intermediate with identical retention times to that shown by 25, was seen in the HPLC analysis. The appearance of this iminium species starts shortly after initiation of the incubation and reaches a maximum after varying time periods, depending on the enzymatic activity of the particular culture. There is a concurrent disappearance of 26. Extraction of the incubation mixture by means of ethyl acetate removes the bisindole metabolites, thereby allowing the iminium intermediate(s) to remain in the aqueous phase. Subsequent borohydride reduction of the latter regenerates 26 thereby establishing that no structural rearrangements had occurred.

It should be noted that in an independent study, Scott and co-workers [13] have also shown the conversion of 26 to vinblastine by cell free extracts of their C. roseus cultured cell line.

As the above studies were underway, a simultaneous research program concerning the enzymatic coupling of catharanthine (17) and vindoline (18) (Fig. 19) was also undertaken. Essentially all of the experiments within this area concern investigations with cell free extracts but, as in the above studies with 26 as substrate, several experiments were performed with "spent" medium.

In brief, the results of enzymatic coupling of 17 and 18 in the "spent" medium parallel those already presented above for the biotransformation of 26. When "spent" medium is obtained from an older growing cell culture for example, 19 days in a recent experiment, significant coupling activity is noted. On the other hand, a younger culture, in which cell lysis is insignificant, does not afford an enzymatically active medium.

Initial experiments involved tritium - labelled catharanthine and radioinactive vindoline in a manner similar to that described with cell free extracts obtained from C. roseus leaves (Fig. 15). The age of cultures, from which such extracts were prepared, varied from 5 - 11 days and involved incubation periods of 3 h. Under these conditions, enzymatic coupling of 24 and 18, presumably via intermediate 25, affords leurosine (27) and other dimeric alkaloids.

In a parallel study with "resting" cells, that is, cells grown in a nutrient medium and then resuspended in MES (morpholinoethanesulfonic acid) buffer, it was shown that the coupling activity was also maintained. For example, cells were initially grown for 10 days in IB5 medium, filtered and resuspended in MES buffer containing 8% sucrose for 44 - 212 h. The cells were then harvested and cell free extracts prepared according to Scheme 1. Experiments with the latter extracts and involving 17 and 18 as substrates, revealed coupling activity to afford leurosine (3% yield) as one of the major bisindole alkaloids isolated.

Extensive investigations with non-radioactive substrates and cell free extracts obtained from various cell cultures were pursued. Varying ages of cultures (5 - 23 days), differenct co-factors (FAD, FMN, NADPH, NADP, ATP/Mg^{+}), and different inorganic salts ($MnCl_2$, $CoCl_2$) have been studied in order to ascertain the effect of such parameters on the enzymatic coupling activity. Similarly, different incubation periods (3 - 30 h) have been evaluated.

In summary, such experiments have revealed the presence of appropriate enzymes in the cell free extracts and responsible for the coupling of catharanthine (17) and vindoline (18) to the bisindole systems, 3',4'-anhydrovinblastine (26), leurosine (27), catharine (28), vinamidine (29, R=H) and hydroxyvinamidine (29, R=OH) as well as other as yet uncharacterized bisindole compounds. Their relative yields depend on the origin of the cell free extract and the incubation conditions involved. A vindoline dimer, probably identical with that obtained by Rosazza et al in their studies on microbial transformation of vindoline[14], has also been isolated.

Studies concerned with the isolation of the relevant enzymes involved in the above biotransformations of 3',4'-anhydrovinblastine as well as those responsible for the coupling of catharanthine and vindoline are presently underway. Enzyme isolation procedures and HPLC monitoring of such crude enzyme mixtures are already described in our earlier studies with C. roseus and with the objectives of producing the alkaloids catharanthine, vinblastine and vincristine by such culture techniques. Related to these objectives, we wish to derive information about their biosynthesis and biotransformation and with future developments in enzyme isolation, ascertain the nature of the enzymes involved. With the availability of cell and/or enzyme immobilization technology, improved methods for possible "commercialization" of such processes may also be developed. Although much remaining data must await further experimentation, we believe that the field of plant tissue cultures has considerable potential in pharmaceutical applications. The area will also provide interesting challenges and avenues of basic research for practicing natural products chemists, plant biochemists, plant geneticists, etc.

ACKNOWLEDGEMENTS

The author serves merely as a spokesman for the enthusiastic and dedicated group of researchers who have made possible the above discussion. The majority of the studies presented have been obtained within a collaborative program between the author's laboratory and the National Research Council of Canada, Plant Biotechnology Institute 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-LaPrairie, S. Rambold and J. Rushkowsky are responsible for development of some of the cell culture lines (953, 200 GW) discussed above and for the large scale propagation of these lines in order to produce the natural products involved. Development of other cell lines discussed occurred in the author's laboratory, as well as development of all the analytical methods (hplc) and isolation and chemical characterization of all the alkaloids discussed. All studies concerning crude enzyme

preparations (cell free extracts), and partially purified enzyme systems were performed at UBC, after enzyme isolation and hplc techniques for protein monitoring and recognition of bisindole metabolites had been established. The extensive chemical synthetic experiments, as required in the biotransformation studies, were also performed at UBC. The research workers involved are: B. Aweryn, J. Balsevich, B. Botta, C. Buschi, L.S.L. Choi, M. Gumulka, G.M. Hewitt, T. Honda, P. Kolodziecjczyk, G.C. Lee, N.G. Lewis, T. Matsui, T. Nikaido, J. Onodera, I. Perez, P. Salisbury, T. Sato, S.K. Sleigh, K.L. Stuart, R. Suen, and B.R. Worth.

Financial aid was provided by a grant to the author from the Natural Sciences and Engineering Research Council of Canada, an NRC Research Contract under the Fermentation Technology Program, (00-310-SX-8-3011) and, most recently, through a collaborative program with Allelix Inc., Mississauga, Ontario, under the NRC/PILP program.

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Received, 8th April, 1986