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STUDIES IN PLANT TISSUE CULTURE

THE SYNTHESIS AND BIOSYNTHESIS OF INDOLE ALKALOIDS

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Abstract—Studies involving plant tissue cultures of *Catharanthus roseus* are described. Investigations concerning the propagation of cell lines of this plant for the purposes of producing indole alkaloids within the Corynanthé, Aspidosperma and Iboga families are presented. The utilization of such tissue culture systems for studies in biosyntheses and isolation of enzymes are also discussed.

Indole alkaloids comprise a large and important family of natural products. Their chemistry, synthetic and biosynthetic studies have maintained the interests of a large number of investigators and span over many years. However, a much more recent development within this area concerns the utilization of plant tissue culture techniques particularly for the purpose of production of selected members, more detailed biosynthetic studies and investigations on enzyme related reactions. Recent advances in biotechnology, for example, cloning methods, have also allowed considerations in research directions not normally available to the earlier investigators. A number of books[1-4] and recent review articles[5,6] provide excellent summaries of the previous studies in the tissue culture area and include investigations from the various laboratories pursuing studies in the indole alkaloid field. This article summarizes our recent experiments with plant tissue cultures of *Catharanthus roseus* and emphasizes the approaches which have been developed within selected aspects of the synthesis and biosynthesis of appropriate indole alkaloids present in such cultures. 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 antitumor 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(**4**) with vindoline afforded an important route to the bisindole system. Figure 1 summarizes our initial studies in which the three bisindole products (**5**–**7**) were isolated. Under optimum conditions, 3′, 4′-anhydrovinblastine (**5**) 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′-anhydrovinblastine(**5**) is also formed enzymatically from catharanthine (**1**) and vindoline (**2**; Fig. 2) and that **5** is subsequently transformed to the alkaloids vinblastine (**10**, R = Me), leurosine (**8**) and catharine (**9**) (Fig. 3).

An independent and simultaneous study by Scott[16] provided results analogous to those outlined in Fig. 2.

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.[17-27]

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.

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 fresh-weight, about 2 cm in dia, 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 to 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

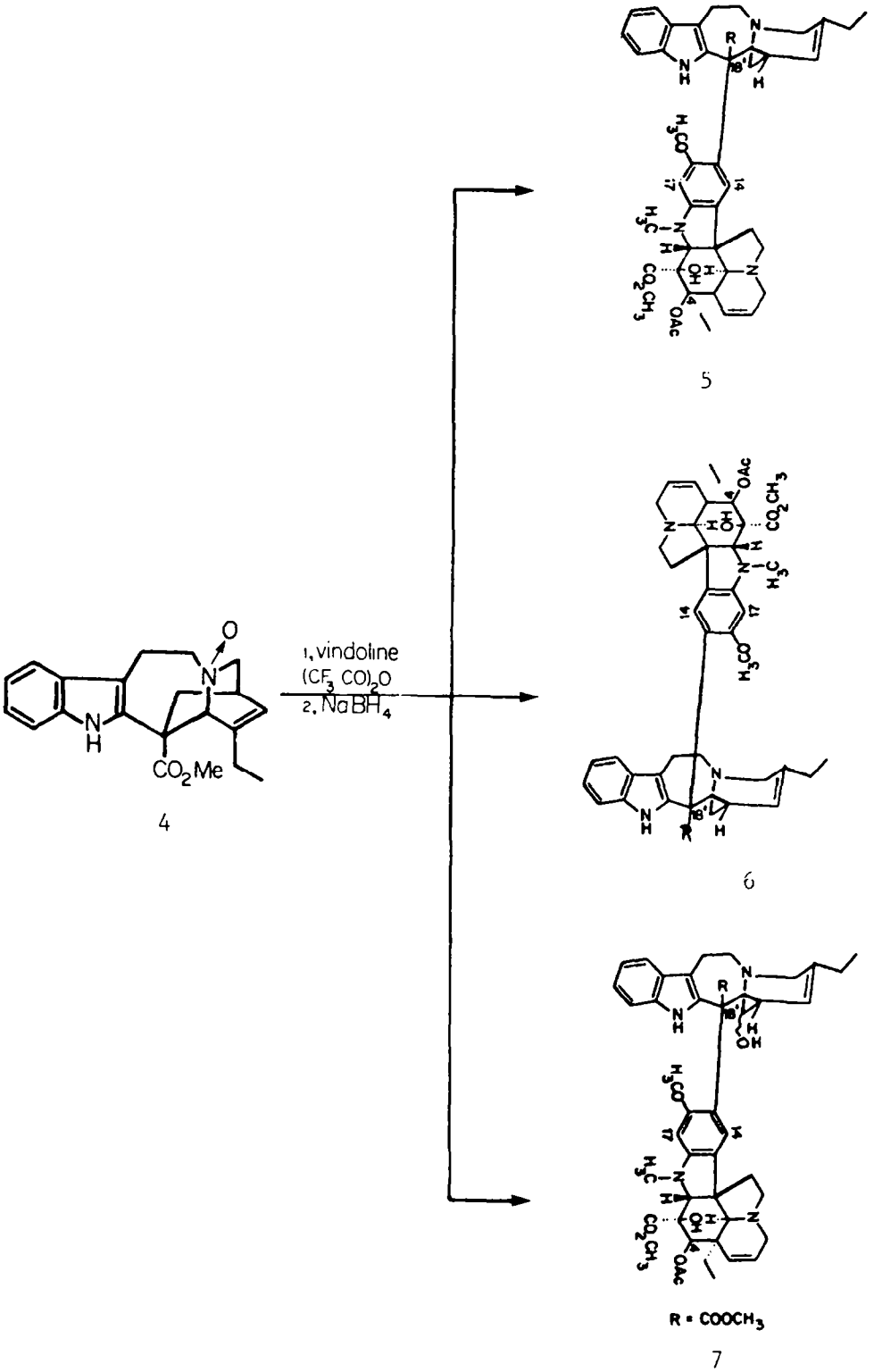

Fig. 1. Summary of results when catharathine N-oxide **4** is coupled with vindoline **2**.

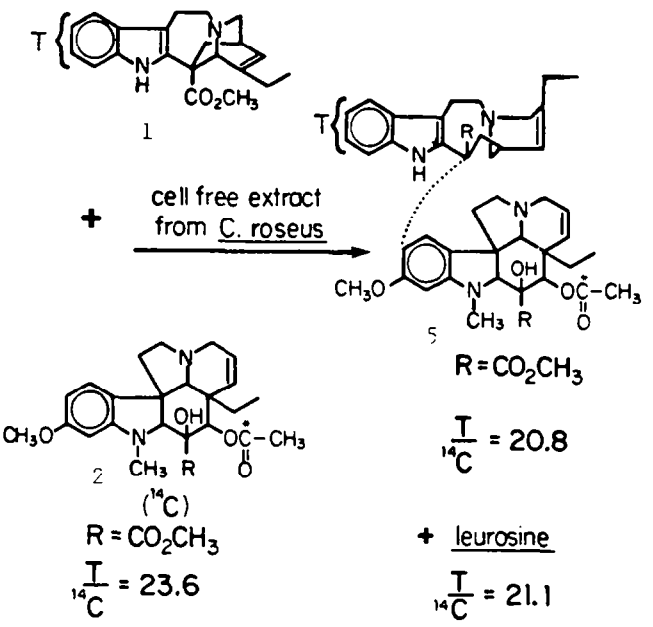

Fig. 2. The biosynthesis of 3′, 4′-anhydrovinblastine **5** and leurosine **8** from catharanthine **1** and vindoline **2** employing cell free extracts.

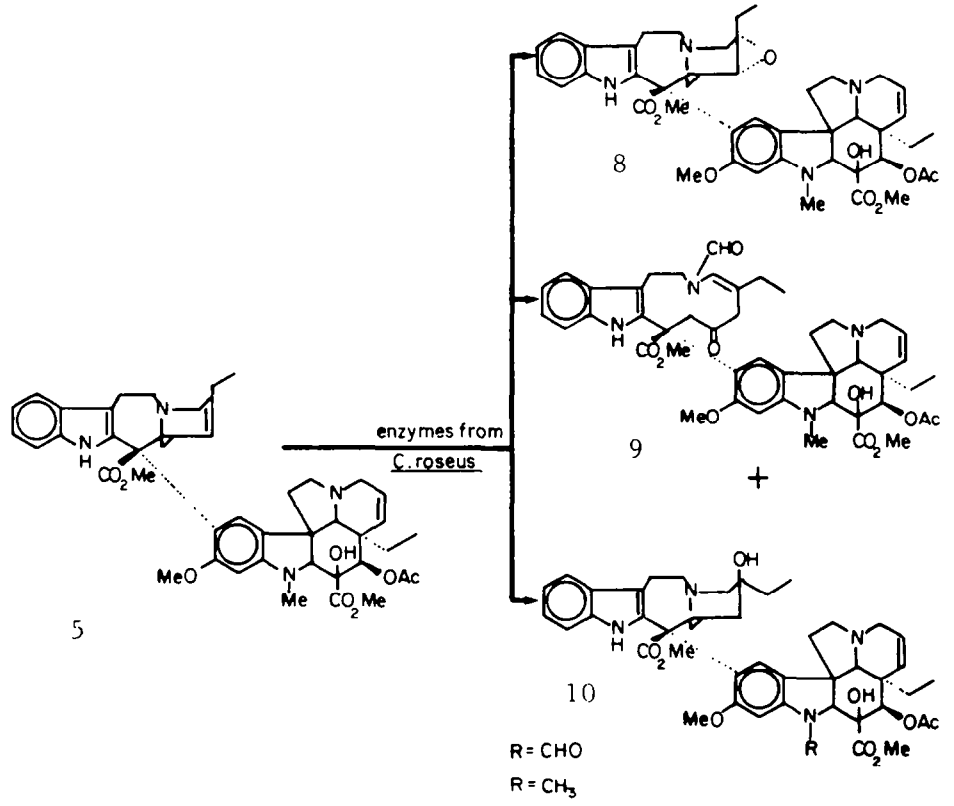

Fig. 3. Enzyme catalyzed conversion of 3′, 4′-anhydrovinblastine **5** to leurosine **8**, catharine **9** and vinblastine (**10**, R = CH_3) employing cell free extracts.

7.5 l. Microferm bioreactors followed essentially the pattern shown in Fig. 4. After incubation with actively growing cell suspension the mitotic index (MI) dropped to zero within 24 hr and remained there for 2–3 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 8–10 while the variation in pH stayed within half a unit.

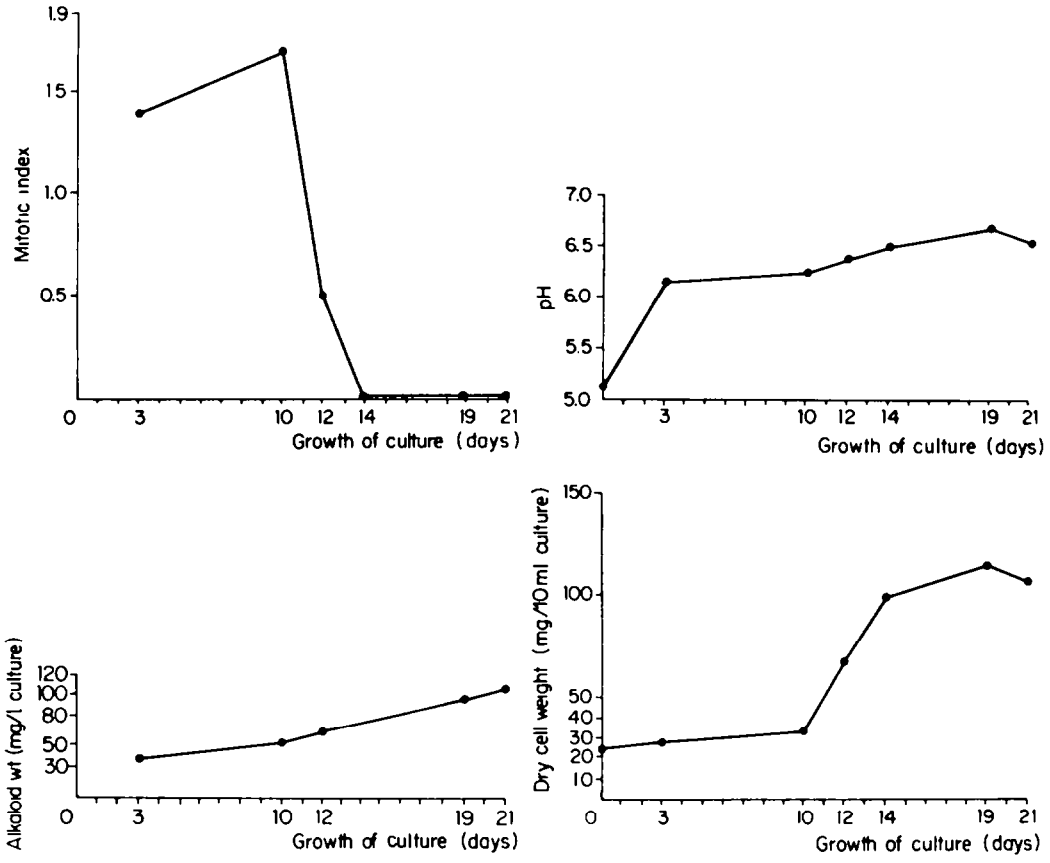

Fig. 4. General growth pattern of *C. roseus* tissue culture in bioreactor.

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 46.13% Strychnos while 56.71% yielded Corynanthé type alkaloids. Only several lines produced catheranthine (1), a member of the Iboga family.

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

The 953 *line.*

Studies with this selected line were performed both in shake flasks and bioreactors employing the 1B5 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

Table 1. Alkaloid yields from batches of 953 line *C. Line* 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

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 (**11**), yohimbine (**12**), isositsirikine (**13**), vallesiachotamine (**14**), strictosidine lactam (**15**), lochnericine (**16**), hörhammericine (**17**), hörhammerinine (**18**), vindolinine (**19**), 19-epivindolinine (**20**), 19-acetoxy-11-methoxytabersonine (**21**), 19-hydroxy-11-methoxytabersonine (**22**) and dimethyltryptamine (**23**).

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. 5. These show that the percentage of alkaloid per gram of cell weight increases with time, with optimum production at 3–4 weeks. Figure 6 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. 7 demonstrates a more rapid increase in the biosynthesis of ajmalicine (**11**) and yohimbine (**12**; Corynanthé family) than observed for vindolinine (**19**; Aspidosperma family). That is, the simple Corynanthé alkaloids ajmalicine (**11**) and yohimbine (**12**)

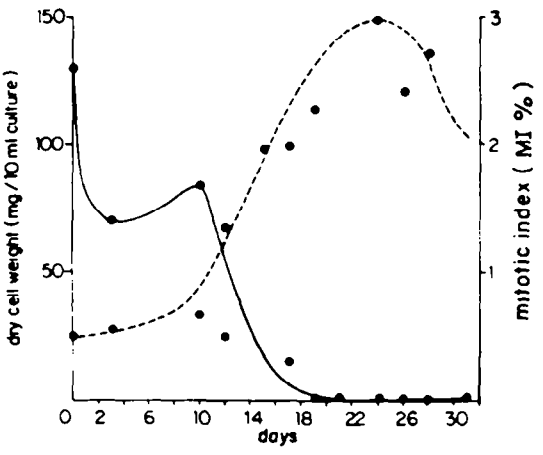

Fig. 6. Dry cell weight (– – – –) and mitotic index (——).

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

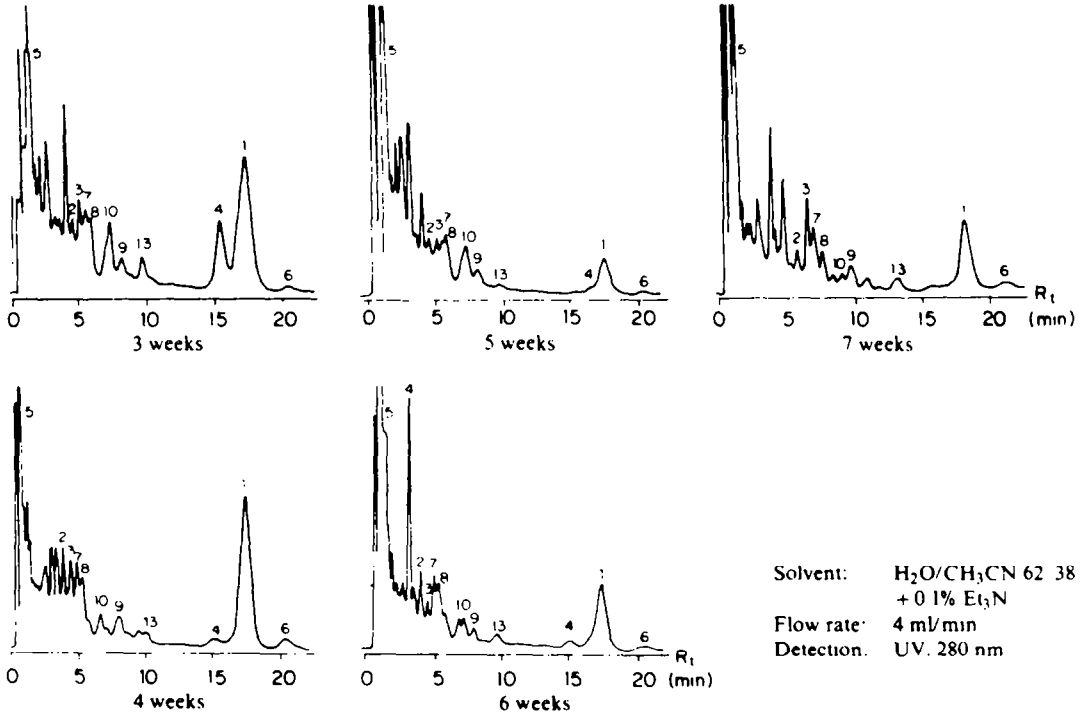

Fig. 5. Reverse phase HPLC of alkaloid mixtures obtained after different growth periods.

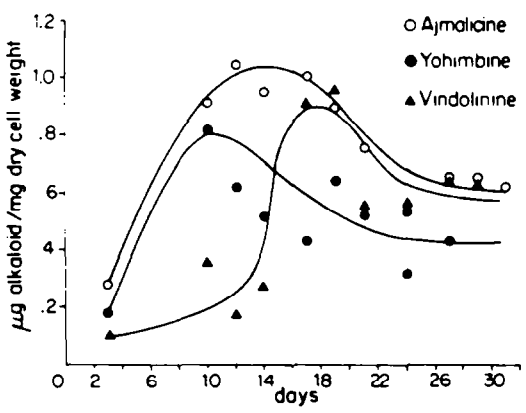

Fig. 7. Content of ajmalicine **11**, yohimbine **12** and vindolinine **19** at the earlier periods of culture growth.

reach maximum concentration at a much earlier period in culture growth than the biosynthetically more complex vindolinine (**19**). These are presumably derived from a common key intermediate, strictosidine (**24**), reflecting the differences in complexity of their biogenesis. Figure 7 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 5 shows HPLC traces of the later stages of growth period (3–7 weeks). Each sample contained ajmalicine (**11**) 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.

The 200GW *line.*

Another particularly interesting cell line under recent investigarion 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 cataranthine (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.

Biotransformation studies. The above discussion and previous publications noted above have demonstrated the capabilities of different tissue culture cell lines from *C. roseus* to produce various types of alkaloids. Another area of potential importance for the purpose of increasing cell yield of desired products, as well as for biosynthetic investigations, concerns the use of selected cell lines for biotransformation of appropriate substrates introduced into the culture medium at various stages of culture growth. Studies involving the transformation of various functional groups within organic compounds by plant tissue culture techniques have been reported[6] but compared to the extensively studied area of microbial transformation much research is still required with such cultures before a proper understanding of this method can be attained. To this end we have initiated some studies[27] with selected *C. roseus* cell lines and appropriate substrates available from our earlier investigations.

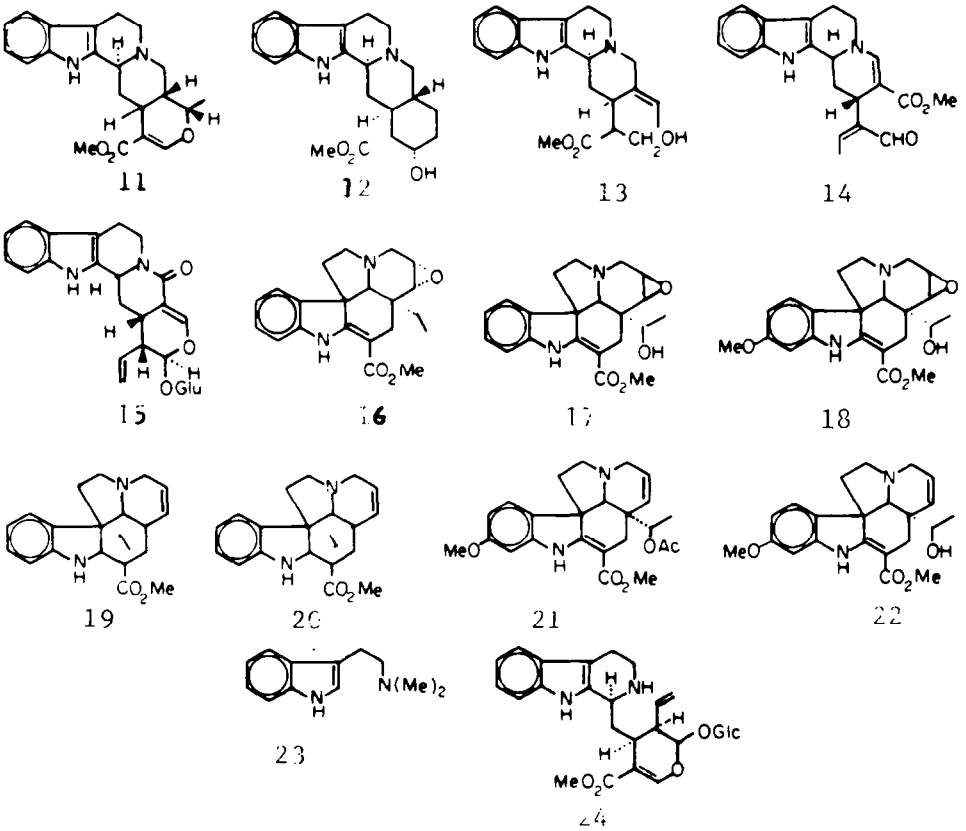

Table 3. Alkaloids isolated from the 200GW cell line

Alkaloid	% Yield from dry cell wt.	% of crude alkaloid mixture
1	0.005	1.35
20	0.015	4.05
epimer of 20	0.026	7.02
11	0.006	1.62
17	0.002	0.54
18	0.005	1.44
19	0.002	0.54
20	0.002	0.54
15	0.224	60.48

% figures refer to isolated yields.

The substrate 3′,4′-anhydrovinblastine (**5**) available from the synthetic route outlined in Fig. 1, was selected for our initial experiments. Several serially cultured cell lines have been propagated for the preliminary screening to determine their capability of biotransforming **5** into desirable products. Only four experiments with each line were necessary to ascertain whether productive biotransformation was occurring. For each line, these experiments were: (a) two control experiments (24 and 72 hr) involving only cells to determine whether alkaloids isolated were being produced by the cells or were metabolites of the precursor, (b) cell line + 5 (3–5 mg), harvested 24 hr after addition of substrate, and (c) cell line + 5 (3–5 mg), harvested 72 hr after addition of substrate. The results of these experiments with a number of established cell lines are given in Table 4.

Peliminary screening of these cell lines demonstrated that several lines were metabolizing 3′,4′-anhydrovinblastine (**5**). However, the determination of metabolic products was complicated since the cell lines were producing their own alkaloids, and these possessed retention times on TLC plates which were very similar to the desired metabolic products. Therefore, for our further studies, we chose a line coded as "916". This *C. roseus* cell line was somewhat unique: it exhibited satisfactory growth characteristics, etc. but did *not* produce any of the alkaloids normally found in the other lines which we have investigated. A discussion about the propagation of this line will be published elsewhere.

In the initial study with the 916 cell line, 3–5 mg of 3′,4′-anhydrovinblastine (**5**) was incubated with the cells in shake flasks for 2, 6, 12, 18, 24, 48 and

Table 4. Small-scale biotransformation studies of 3′, 4′-anhydrovinblastine **5** in *C. roseus* suspension cell cultures (shake flasks)

Cell line	Sample[a)b)]	Incubation time (h)	Weight of freeze-dried cells (sample) (g)	Weight of basic alkaloid fraction (mg)	% of 5 in basic alkaloid fraction
953[23]	Control	24	11.6	6	-
953	5	24	11.1	4	<1
953	Control	72	9.5	28	-
953	5	72	11.1	9	0
943[18]	Control	24	10.0	9	-
943	5	24	11.4	17	0
943	Control	72	10.6	9	-
943	5	72	10.6	48	<1
340YG[21]	Control	24	10.4	8	-
340YG	5	24	10.9	25	0
340YG	Control	72	10.1	11	-
340YG	5	72	11.0	17	0
200GW[24]	Control	24	11.2	38	-
200GW	5	24	10.6	43.8	<1
200GW	Control	72	11.2	5.4	-
200GW	5	72	10.3	13	0
Growth medium	+ 5	24	12.9	4.5	0
Growth medium	+ 5	72	13.6	2.3	0

a) Control means normal cell propagation without 5.

b) In all experiments, 5 was added as hydrogensulfate salt.

Table 5. Small-scale biotransformation studies of 3′, 4′-anhydrovinblastine **5** in *C. roseus* suspension cell cultures (shake flasks), cell line 916

Incubation time (h)	Weight of freeze-dried cells (g)	Weight of basic alkaloid fraction (mg)	Percent of 5 in basic alkaloid fraction (by HPLC. and TLC.)
2	7.24	21	15
6	9.00	17	0
12	4.80	12	0
18	6.11	15	0
24	6.60	12	0
24	5.48	16	0
48	4.80	15	0
48	2.90	11	0
72	5.40	6	0
72	5.02	11	0

72 hr. The results of this study are summarized in Table 5. In the sample incubated for 2 hr, mainly **5** was found; samples incubated for 6–72 hr contained a new, less polar compound. However, the highest concentration of this new product was observed in 24- and 48-hr-incubation samples. In 72-hr-incubation samples the concentration of the new product was decreasing and degradation products appeared. From these samples the new compound was subsequently isolated by TLC and HPLC methods. The initially isolated amount of material was sufficient to determine the dimeric nature of this product (UV: λmax at 310, 290 and 160 nm) and its molecular weight (*m/z* 822.3819), which corresponds to the formula $C_{46}H_{54}N_4O_{10}$. Further large scale experiments (3′ 4′-anhydrovinblastine, 300 mg added as the hydrogensulfate salt) involving an incubation time of 48 hr were performed in a *Microferm* bioreactor (cell line 916, 5.5 l.) and allowed a more detailed study of the biotransformation process.

The results of this experiment are summarized in Tables 6 and 7. Based on the amount of recovered substrate, the transformation of 3′, 4′-anhydrovinblastine (**5**) to leurosine (**8**) and catharine (**9**) was 25.5 and 16.3% respectively, or approx. 42% of **5** had been utilized by the cells. It should be noted, however, that no attempts have yet been made to optimize the yields of specific products.

The results also indicate that these high-molecular-weight alkaloids have passed through the cell walls

Table 6. Biotransformation of 3′, 4′-anhydrovinblastine (**5**, 300 mg[a]) in *C. roseus* suspension cell culture in *Microferm* bioreactor, cell line 916, 48 hr

Sample	Weight of sample (g)	Weight of methanol extract (g)	Weight of neutral ethyl acetate extract (g)	Weight of basic ethyl acetate extract (g)
Cell material	10.25	3.5	0.469	0.065
Supernatant	ca. 140	-	0.143	0.187

[a]) Added as the hydrogensulfate salt

Table 7. Alkaloids isolated from biotransformation of 3′, 4′-anhydrovinblastine (**5**, 300 mg[a]) in *C. roseus* suspension cell culture in *Microferm* bioreactor, cell line 916, 48 hr

Alkaloid isolated	Weight of alkaloid isolated (mg)					% of the substrate added
	Basic super-natant extract	Basic cell material extract	Neutral super-natant extract	Neutral cell material extract	Total	
3′,4′-anhydro-vinblastine (5)	-	30.0	16.4	54.0	100.4	33.5
Catharine (9)	28.8	3.1	0.8	-	32.7	10.9
Leurosine (8)	17.0	8.2	17.2	8.6	51.0	17.0

[a]) Added as the hydrogensulfate salt

since bisindole alkaloids were present in both the cell material and the culture medium.

Finally, the short period of time required for such biotransformations (24–48 hr) is interesting, particularly when compared to plant cell culture production of alkaloids from nutrients present in the growth medium (usually several weeks). The inoculation of suspension cultures with biosynthetically "advanced" precursors which reduce time periods for the production of target compounds may provide an important avenue for the commercial production of such pharmaceutically important agents. Further studies are underway.

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.

Brief mention has already been made (Figs. 2 and 3) 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 Fig. 8.

The crude enzyme thus obtained was utilized in the experiments portrayed in Figs. 2 and 3 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 (**25**) and secologanin (**26**) to **2** (Fig. 9).[28]

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 3′,4′-anhydrovinblastine (**5**) and its subsequent transformation to the other bisindole alkaloids (Figs. 2 and 3). Thus we have initiated a study directed at the recognition and purfication of the relevant enzyme(s) involved in this coupling reaction.

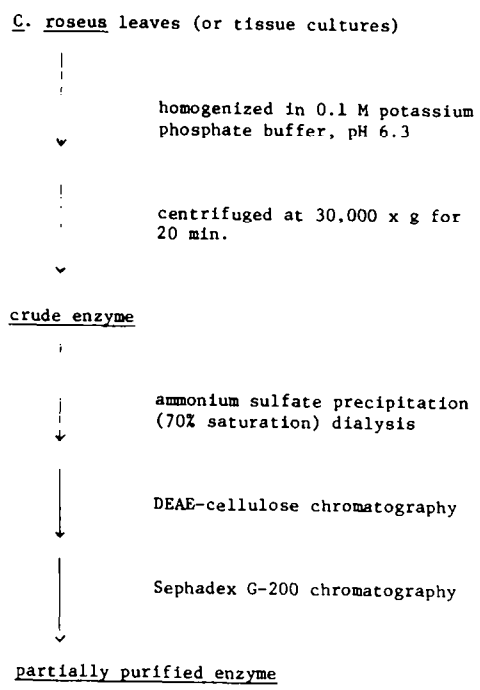

Fig. 8. General procedure for preparation of cell free systems from *C. roseus* leaves and/or tissue cultures.

The coupling enzyme activity was determined by monitoring the formation of 3′,4′-anhydrovinblastine (**5**) and leurosine (**8**) using radio-labelled 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 8 lists the retention times for the respective protein under the conditions used for the analyses of the cell free extracts.

Figure 10 showed the HPLC profile of the crude enzyme from *C. roseus* leaves as prepared according to the procedure outlined in Fig. 8. It will be noted that a mixture of proteins varying in molecular weights of approx. 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 separate the cell free extract (crude enzyme) by precipitation, dialysis and chro-

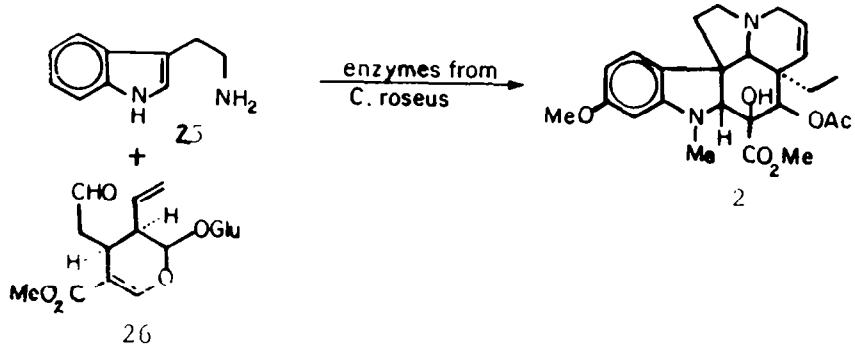

Fig. 9. The enzyme catalyzed synthesis of vindoline **2** from tryptamine **25** and secologanin **26**.

Table 8. 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

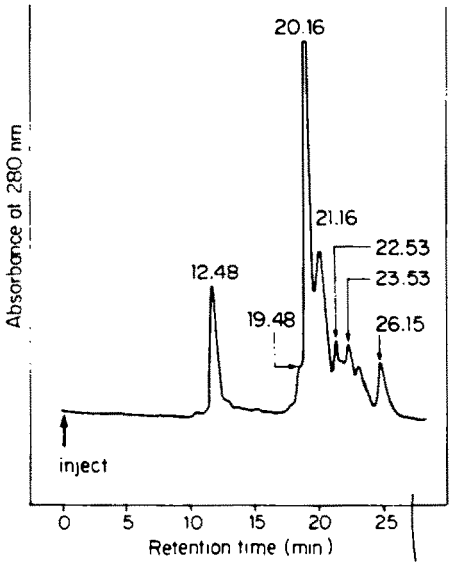

Fig. 10. HPLC analysis of cell free extract prepared from *C. roseus* leaves.

matographic techniques to a "partially purified" enzyme stage (Fig. 8).

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 nM, pH 6.8). The elution profile of the DEAE-cellulose chromatography is shown in Fig. 11.

Fractions 21–30 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 (Fig. 12). The fractions corresponding to the

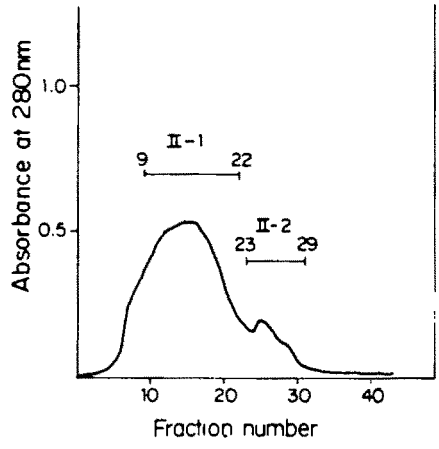

Fig. 12. Elution profile of Sephadex G-200 chromatography—Fraction II.

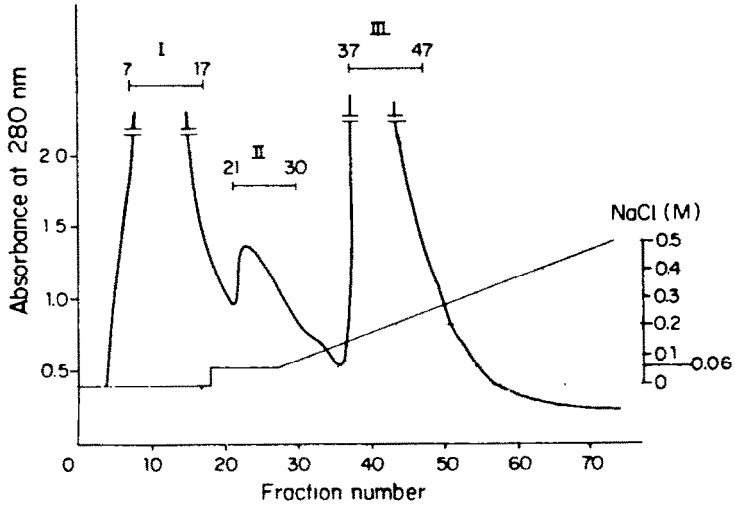

Fig. 11. Elution profile of DEAE cellulose chromatography of cell free extract from *C. roseus* leaves.

two peaks II-1 (fractions 9 to 22) and II-2 (fractions 23–29) were collected and analysed by HPLC (Fig. 13) 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 Table 9) corresponding to elution peaks of different retention times were collected and the results of their coupling enzyme activity determination are shown in Table 9.

It is clear from these investigations that the enzyme system(s) involved in the biosynthesis of **5** and **8** from the appropriate monomeric alkaloids are present in the short HPLC retention time region (11–20 min, Fraction A in Table 9). From the calibration standards (Table 8) 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 14 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 min 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. Further studies are underway in this area.

In summary, this article 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.

EXPERIMENTAL

The procedures described below are most pertinent to the discussion above. Further details of the large number of experiments performed, isolation and characterization of alkaloids, etc are provided in ref. 13–28.

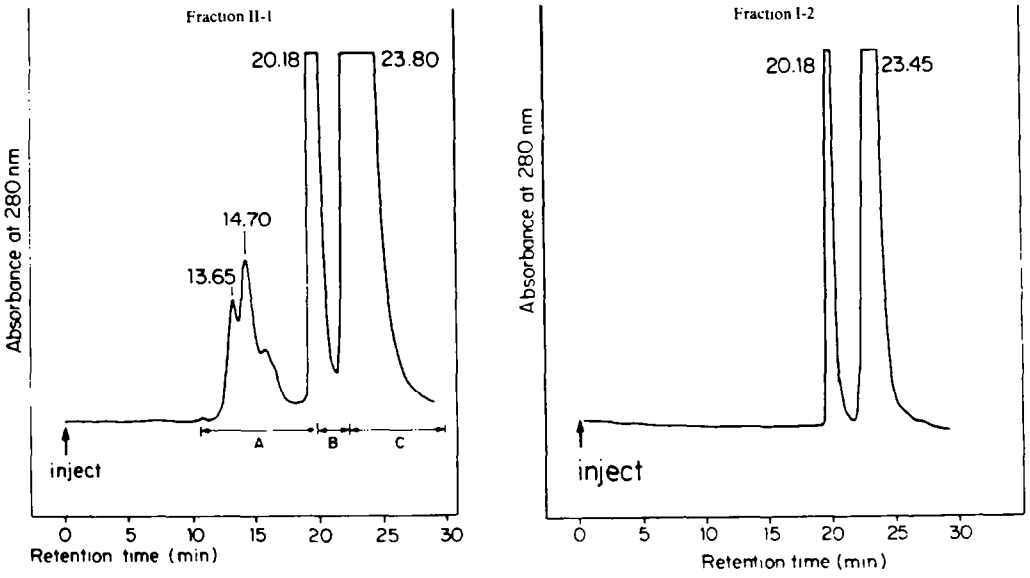

Fig. 13. HPLC profiles of fractions II-1 and II-2 from Sephadex G-200 chromatography.

Table 9. Preparative HPLC—enzyme fraction II-1 and evaluation of coupling enzyme activity

Fraction	Retention time (min)	Net activity (dpm)	
		3',4'-anhydrovinblastine	Leurosine
Frs. A	11 - 20	4137	3012
Frs. B	20 - 22.5	57	965
Frs. C	22.5 - 30	0	0

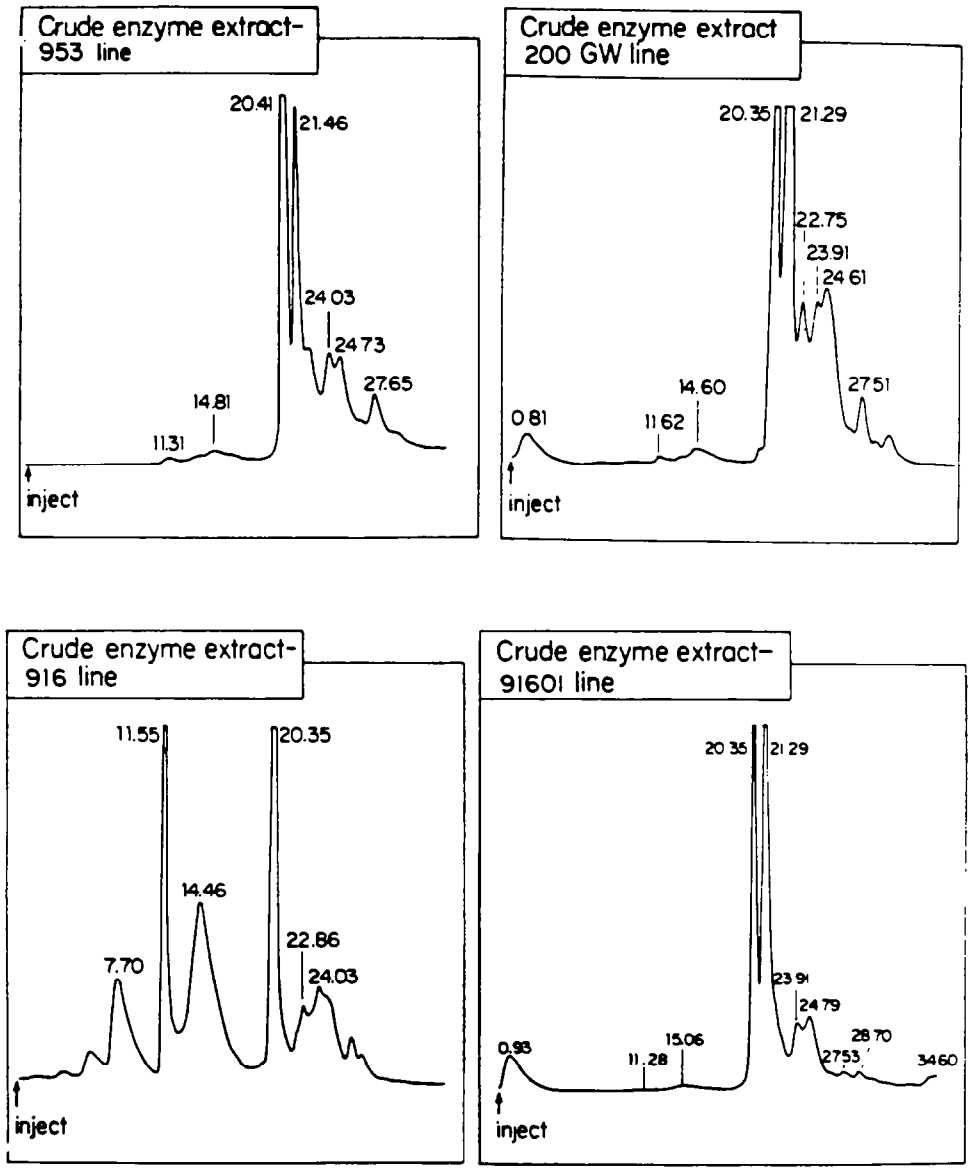

Fig. 14. HPLC profiles of crude enzyme extracts from several *C. roseus* tissue culture cell lines.

Cell cultures. Seeds of periwinkle (*Catharanthus roseus* (L.) Don.) were planted in a mixture of Vermiculite[R], peat and sand, supplemented with Osmocote[R] fertilizer pellets and grown in a greenhouse in photoperiods of 16 hr/day of daylight and, if necessary, white fluorescent and incandescent light of 40 $W.m^{-2}$. As soon as the plants had developed 2 pairs of leaves, they were potted and transferred to a growth room at 26° at day and 22° at night with white light, 20 $W.m^{-2}$, in photoperiods as before. Prior to flowering, cuttings were treated with 0.1% EMS (ethanesulfonic acid methyl ester) in test tubes over a period of 4 days. The tubes were replenished daily. Thereafter the cuttings were harvested, the inflorescences bagged and stored in a refrigerator at 4–6° for 2 days.

These inflorescences were then sterilized by immersion in 70% EtOH for 10 min and washed with sterile, dist. water. Buds, approx. 1.5–2.0 mm in length, were selected, and anthers removed under a dissecting microscope. The anthers were incubated, 10 per 2 ml, floating on liquid 0.1 MSC medium, i.e. medium after Murashige and Skoog[9] fortified with 0.1 mg/l NAA (α-naphthalene acetic acid) and 1 g/l casein hydrolyzate (NZ-Amine, Humko–Sheffield Co., Norwich, New York) in 60 × 15 mm petri-dishes. The dishes were sealed with Parafilm[R] and stored in plastic boxes in dim light at 27°. Within 3–4 weeks *ca* 30% of the anthers formed greenish callus perferentially at the anther wall.

Some of the anthers produced white callus which burst from the mass of microspores. Karyological investigation revealed dividing haploid cells. Derivatives of the daughter cells however, were found to be diploid and approx. 5% polyploid. Sometimes tracheids formed in abundance. In view of the variability of cell lines, as observed later, no attempt was made to select for haploid callus.

After 6–8 weeks individual calli were cloned by transfer to 30 ml of nutrient medium solidified with 0.8% agar in jars. Once the calli had grown to a clump of cells *ca* 2 cm in dia, a few small pieces were subcultured on agar medium, the rest subcultured in 50 ml of liquid Gamborg's B5 medium[10] and continuously shaken. Subcultures on agar medium were kept at 27° in continuous light, 10 $W.m^{-2}$, and transferred to fresh medium at monthly intervals.

Alkaloid synthesis. To test the different cell lines for their ability to synthesize indole alkaloids, subcultures were transferred to 500 ml Erlenmeyer flasks containing 250 ml of

alkaloid production medium, and agitated on gyratory shakers (130 rpm) at 27° in continuous light 10 $W.m^{-2}$. Samples were withdrawn at weekly intervals and analyzed for their alkaloid content.

In order to obtain large amounts of cell material from cell lines containing alkaloids of interest, cultures were grown in alkaloid production medium in 7.5 l. Microferm–Bench top bioreactors (New Brunswick Scientific Co. Inc., Edison, N. J., U.S.A.) equipped with sintered disc spargers (5 cm). To minimize the shearing effect, the bioreactors were stirred at 200 r.p.m. and aerated with 35 ml min^{-1} air per liter culture. Samples were taken every second day and analyzed for mitotic index (MI), pH, and dry weight. The alkaloid content was analyzed at irregular intervals, but with increasing frequency after the mitotic index started to decline.

Procedures for culture growth. The two procedures described below are typical conditions employed for the large scale propagation of cultures in bioreactor and shake flasks.

Growth in bioreactor. The innoculum was grown up in 1B5 medium in shake flasks over a period of approx. 4 days. To 5 l. of Zenk's alkaloid production medium contained in a 7.5 l. Microferm bioreactor, 500 ml of inoculum was added. Under agitation at 200 r.p.m. and aeration of 35 ml of air per l. culture per min and at a temperature of 26° the growth of the cultures was allowed to proceed for 2 weeks. Samples were withdrawn at various intervals and analyzed for mitotic index ((MI), dry cell weight and pH. The results of these analyses are given in Table 10.

Employing the procedure described above another 500 ml of inoculum was introduced into a 7.5 l. bioreactor and the growth was allowed to proceed for a 2-week period. As before the sample analyses were taken and the data is presented in Table 11.

Harvesting of the cells after the 2-week period provided 145 g of freeze-dried cells.

Growth in shake flasks. A small piece of callus was added to a 250 ml Erlenmeyer flask containing 100 ml of 1B5 medium and shaken for 5 days on a gyratory shaker (130 r.p.m.) at 27° in continuous light. After this period the resulting cell suspension was transferred to a 500 ml Erlenmeyer flask containing 250 ml of Zenk's alkaloid production medium and agitation continued for a further 3 weeks. Harvesting provided 95 g of freeze-dried cells.

Standard extraction procedure. A typical extraction of freeze dried cells was carried out as follows: freeze dried cells (7.8 g) were suspended in MeOH (100 ml) and extracted using an ultrasonic bath for 4 hr. The suspension was filtered and the solid re-extracted (2 × 100 ml). The extracts were combined and concentrated *in vacuo*. The residue was suspended in 1N HCl (30 ml) and washed with EtOAc (4 × 30 ml). The aqueous soln was neutralised ($NaHCO_3$), the pH adjusted to *ca.* 9.5 (1N NaOH) and extracted with EtOAc (4 × 30 ml). The combined extracts were dried and concentrated to afford the crude alkaloids (35 mg). This mixture was further fractionated by trituration to give 25 mg of CH_2Cl_2 soluble material. The residue (soluble in MeOH) contained very few detectable, and only in one case isolable alkaloids.

Alkaloid isolation from bioreactor samples

A typical procedure is as follows. The crude CH_2Cl_2 soluble fraction (180 mg) was chromatographed over Porasil

Table 10.

Day	MI (%)	Dry Weight mg/10 ml culture	pH
0	0.1	12.6	
1	0	13.5	
2	0	14.3	6.04
3	0	15.4	6.0
6	1.8	25.4	6.16
8	1.3	30.6	5.87
10	1.5	30.0	6.07
13	1.2	78.1	5.89
17	1.1	113.0	6.03
20*	0	116.0	6.38
21	0	122.4	6.28

Table 11.

Day	MI(%)	Dry Weight mg/10 ml culture	pH
0	0.6	14.9	5.01
3	0.5	22.0	6.28
7	0.5	49.9	6.17
10	0.1	74.3	
14	0	81.3	6.35

* At this point, L-Tryptophan (500 mg) and sucrose (5 g) were fed continuously over a 24 hour period. After one day (day 21) the culture was harvested to provide 114 gm of freeze-dried cells.

B (150 g, Waters Associates) in a stainless steel column (30 × 2.5 cm) with H_2O/CH_3CN (68/32) containing 0.1% Et_3N modifier, at a flow rate 18 ml/min. A total of 40 × 25 ml fractions were collected and the column then eluted with CH_3CN (400 ml, fraction 41). The 41 fractions were analysed by HPLC (Reverse phase packing, H_2O/CH_3CN, 62/38 containing 0.1% Et_3N at 4 ml/min, detection at both 254 and 280 nm.

Purification of individual alkaloids by chromatographic methods and their characterization is published elsewhere.

Biotransformation studies—Studies with the 916 cell line

(*a*) *Experiment in which cells and medium were processed together.* Compound **5** (500 mg, added as the hydrosulfate salt), was incubated with the 916 line of *C. roseus* cell suspension culture (5.5 *l.*) in a Microferm bioreactor for a period of 24 hr. This provided a freeze-dried sample containing cells and medium constituents of 152.2 g. Extraction with MeOH followed by acid-base partition of the crude MeOH extract according to the standard extraction procedure described above, afforded a crude alkaloid fraction (520 mg). This was subjected to semi-preparative HPLC purification over silica gel (*Waters Associates*) in a stainless steel column (30 × 2.4 cm) using CH_2Cl_2/MeOH solvent system (gradient program) containing 0.1% Et_3N modifier, at a flow rate 15 ml/min. A total of 40 × 50 ml fractions were collected and the column eluted with MeOH. The fractions were analyzed by analytical HPLC using *Waters* Radial-Pak A C-18 cartridge (reverse phase) with MeOH/H_2O (70/30) containing Et_3N at 1.4 ml/min and 4 ml/min, detection at both 254 and 280 nm. A dimeric alkaloid fraction (396 mg) was obtained. Trituration with cold MeOH gave a residue consisting mainly of two components which are separated by preparative plate chromatography using pre-coated TLC plates (silica gel 60 F_{254}), size 20 × 20 cm, layer thickness 2 mm, developed 2 times in solvent system 85% CH_2Cl_2 + 15% MeOH + 0.01% Et_3N. Components were extracted from silica gel employing an ultrasonic bath and using solvent mixture: 75% CH_2Cl_2 + 25% MeOH + 0.01% Et_3N. The two components were identified as **5** (139 mg) and **8** (83 mg) by direct comparison of their spectral and physical properties with that of authentic samples. The mother liquor from the trituration was further purified by preparative plate chromatography, as described above, to afford more **5** (25.6 mg) and **8** (23 mg) plus a less polar component, **9** (29.7 mg). The structure of the latter was assigned on the basis of its spectral properties and confirmed by direct comparison with an authentic sample. Two other minor components exhibiting "dimeric" alkaloid UV absorption were also isolated. Complete elucidation of their structures has not been possible because of the limited amount of material available.

(*b*) *Experiment in which the cells were separated from the growth medium.* **5** (300 mg, added as the hydrosulfate salt), was incubated with the 916 cell line of *C. roseus* cell suspension culture (5.5 l.) in a microferm bioreactor for a period of 48 hr. Cell material and culture medium were separated by filtration and freeze-dried separately. This provided 10.25 g of freeze-dried cells and ~140 g of freeze-dried supernatant (growth medium). Freeze-dried cells were extracted according to the standard extraction procedure described above. This afforded EtOAc neutral extract (469 mg) and EtOAc basic extract (65 mg). The freeze-dried supernatant sample was suspended in 1 N HCl (100 ml) and washed with EtOAc (4 × 75 ml). The combined EtOAc extracts were washed with brine, dried and concentrated to afford the EtOAc neutral extract (143 mg). The aqueous soln was neutralized with $NaHCO_3$, the pH adjusted to 9.5–10.0 (1 N NaOH) and extracted with EtOAc (4 × 75 ml). The combined extracts were washed with brine, dried and concentrated to afford the EtOAc basic extract (187 mg). An intermediate scale HPLC system was employed for the separation of these extracts. A typical procedure is as follows. The crude EtOAc extract was chromatographed over C-18 reverse phase packing in a stainless steel column (30 × 2.5 cm) with MeOH/H_2O (solvent gradient program) containing as modifier 0.1% Et_3N, at a flow rate 6 ml/min. A total of 40 fractions (50–75 ml) were collected for each separation and the column was then eluted with MeOH (300 ml). All fractions were weighed and analysed by analytical HPLC using *Waters* Radial-Pak A C-18 cartridge (reverse phase) with MeOH/H_2O (70/30) containing 0.1% Et_3N at 1.4 and 4 ml/min, detection at both 254 and 280 nm. TLC analyses were performed on analytical KC18F reverse phase pre-coated plates (200 μ thickness) with MeOH/H_2O (70/30. 80/20, 90/10) as solvent. Fractions having the same HPLC profile were combined together and further purified by preparative plate purification using 20 × 20 cm plates coated with silica gel, 1 mm layer thickness, solvent system CH_2Cl_2/MeOH (solvent gradient). Components were extracted from silica gel using solvent mixture: 75% CH_2Cl_2 + 25% MeOH + 0.01% Et_3N.

The chromatography of the basic supernatant extract yielded the following alkaloids: **9** (28.8 mg) and **8** (17.0 mg).

The chromatography of the cells basic extract gave: **5** (300 mg), **9** (3.1 mg) and **8** (8.2 mg).

The chromatography of the supernatant neutral extract gave: **5** (16.4 mg), **9** (0.8 mg) and **8** (17.2 mg).

Preparative plate chromatography of the cells neutral extract afforded: **5** (54.0 mg) and **8** (8.6 mg).

Preparation of cell free extracts. Leaves (5 g) from mature *Catharanthus roseus* plants, grown under greenhouse conditions, were homogenised with 50 ml of Tris-maleate buffer (0.05 M, pH 7.0) or phosphate buffer (0.1 M, pH 6.3) at 4°. Polyclar (5 g) was added portionwise during the homogenisation process. The homogenate was filtered through cheesecloth and then centrifuged at 30,000 g for 20 min. The supernatant (~25 ml) (crude enzyme extract in Fig. 8) was used for various incubation experiments described below. A similar procedure was employed to prepare cell free extracts from the cell cultures as produced by the methods discussed above.

HPLC Analysis of cell free extracts. The HPLC system used was a Waters Associates ALC 100 modified to incorporate a 440 uv detector and Data Module using detection at 280 nm. Separation was achieved employing two protein columns (Waters Associates I-250 and I-125) with potassium phosphate buffer (0.05 M, pH 6.3) as solvent at a flow rate of 1 ml/min. Standard proteins of various molecular weight were used for calibration, and the results are shown in Table 8. Figure 10 showed the HPLC profile of the crude enzyme preparation using the same conditions.

Ammonium sulphate precipitation. The crude enzyme preparation was brought to 70% saturation with solid ammonium sulphate. After stirring for 30 min the mixture was centrifuged at 30,000 g (20 min). The ppt formed was dissolved in potassium phosphate buffer (20 mM, pH 6.8) and dialysed against the same buffer.

DEAE-cellulose chromatography. The above dialysate (30 ml) was applied on a DEAE-cellulose column (2.5 × 10 cm) equilibrated with potassium phosphate buffer (20 mM, pH 6.8). Elution (flow rate 48 ml/hr) with the same buffer (100 ml + 100 ml with 0.06 M NaCl) was followed by a linear solvent gradient from 0.06 M NaCl to 0.5 M NaCl in the same phosphate buffer. Fractions (75 × 7 ml) were collected and analysed for coupling enzyme activity. The elution profile as monitored by the absorbance at 280 nm is shown in Fig. 11. Fractions 21–30 (Peak II) were found to possess the coupling enzyme activity and were concentrated by ultrafiltration (Amicon PM-10).

Sephadex G-200 chromatography. The above concentrate (5 ml) was applied onto a Sephadex G-200 column (1.5 × 4.0 cm) equilibrated with potassium phosphate buffer (50 mM, pH 6.3, containing 1 mM EDTA) and eluted with the same buffer at a flow rate of 7.5 ml/hr. Fractions (40 × 2 ml) were collected and analysed for coupling enzyme activity. The elution profile as monitored by the absorbance at 280 nm is shown in Fig. 12. The HPLC profiles of the two

peaks II-1 (fractions 9–22) and II-2 (fractions 23 29) are shown in Fig. 13.

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