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SYNTHESIS OF DIMERIC INDOLE ALKALOIDS BY CELL FREE EXTRACTS FROM CELL SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; tissue culture, enzymatic synthesis, indole alkaloids; vinblastine, 3′,4′-anhydrovinblastine

Abstract—Crude enzyme obtained from suspension cultures of *Catharanthus roseus* catalysed the coupling of vindoline and catharanthine to form dimeric indole alkaloids. Substantially improved yields were realized through the use of flavin mononucleotide and manganous ion in the reaction mixture. With the optimized conditions, *ca* 25% of the substrates were converted to 3′,4′-anhydrovinblastine.

INTRODUCTION

Vinblastine and vincristine, dimeric indole alkaloids from *Catharanthus roseus*, are proven clinical drugs for the treatment of various leukemias, Hodgkin's disease and solid tumours. These alkaloids are among the most expensive drugs because of their relatively low abundance in intact plants. It was our objective to study the enzymatic synthesis of dimeric indole alkaloids from vindoline and catharanthine, two major alkaloids in *C. roseus* plants (Fig. 1).

In vivo and *in vitro* studies with *C. roseus* have shown that vindoline and catharanthine can be coupled to 3′,4′-anhydrovinblastine [1–3], and the latter, in turn, was convertible enzymatically to leurosine, catharine, and vinblastine [4]. However, the overall yield of coupling products reported was low (0.9%) [1]. *In vivo* feeding of catharanthine and vindoline to seedlings resulted in a yield of 2.6% [2].

We report here on the detection and optimization of the enzyme system involved in coupling of vindoline and catharanthine in cell suspension cultures of *C. roseus*. Yields of coupling products were remarkably enhanced by optimizing the reaction conditions.

RESULTS AND DISCUSSION

Effects of cofactors and metal ions

The effect of various cofactors on the coupling reaction was examined. Those tested were: NAD, NADH, NADP, FMN, FAD, ATP, ADP, AMP and cAMP. Only the riboflavin derivatives, FMN and FAD, gave detectable amounts of the dimeric alkaloids, leurosine and catharine, with a total conversion yield of 1–2%.

Effects of metal ions on coupling in the presence of FAD were also studied (Table 1). Substantial amounts of vinamidine and 3-(*R*)-hydroxyvinamidine, oxidative products of 3′,4′-anhydrovinblastine, were detected upon addition of manganous chloride ($MnCl_2$) to the reaction mixture. The total yield of these products was 10–15%, which was *ca* 10 times higher than the control value (without the metal salt). Manganous ions rather than chloride ions seemed to be responsible for this stimulation because other chloride salts did not enhance the yield. Ammonium, calcium, copper and nickel ions, at the concentrations used, showed inhibitory effects on coupling.

Optimization studies for dimeric alkaloid production were carried out by using either FAD or FMN and $MnCl_2$. In the presence of $MnCl_2$, both FMN and FAD showed similar effects on dimer formation. In both cases the main products were vinamidine and 3-(*R*)-hydroxyvinamidine. FMN was adopted for further studies, and the optimum concentration for coupling was from 0.5–2.0 mM (Table 2). The decrease in yield at a high concentration of FMN (5 mM) might have resulted from the drop in pH (to 6.2).

Effects of varying $MnCl_2$ concentrations were also examined (Table 3). A concentration of 1 mM was optimal since higher concentrations did not further enhance the amounts of dimeric alkaloids formed.

Time course of enzyme reaction

A typical time course of dimeric alkaloid formation is shown in Fig. 2. During the early periods of incubation

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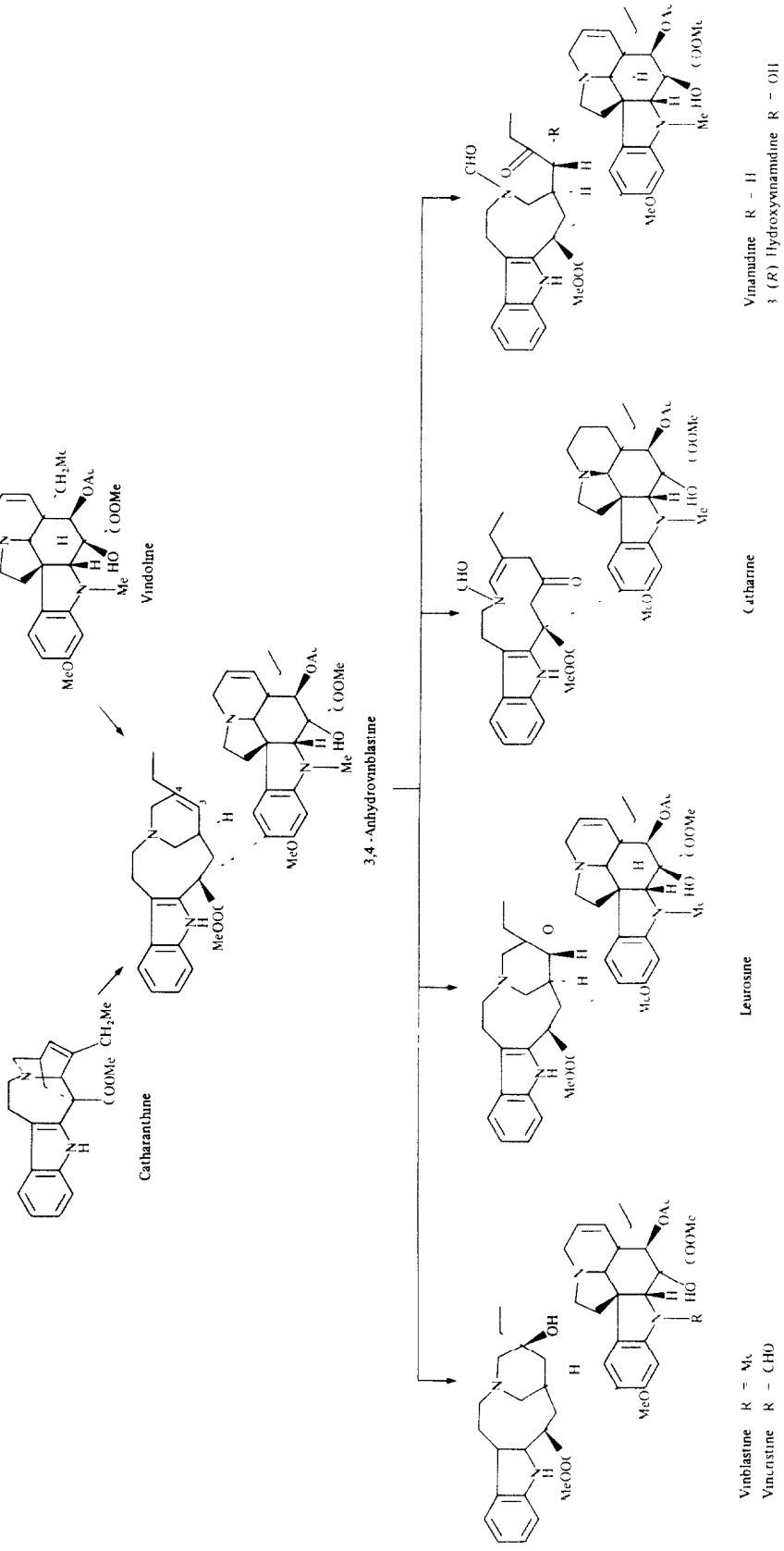

Fig 1 Biosynthetic pathway of dimeric indole alkaloids

Table 1 Effects of metal ions on dimer formation

Metal salt	Concn (mM)	Relative yield of dimers (%)
Control	—	100
NaCl	300	82
KCl	220	71
$(NH_4)_2SO_4$	130	49
$CaCl_2$	10	43
$MgSO_4$	10	143
$FeSO_4$	1	90
$CoCl_2$	1	180
$ZnSO_4$	1	71
$CuSO_4$	1	47
$NiCl_2$	1	48
$MnCl_2$	1	1120

The reaction mixture contained 0 5 mM FAD Amounts of total dimers (sums of amounts of vinamidine, 3-(*R*)-hydroxyvinamidine, leurosine, and catharine) are expressed relative to the control (no metal salt added) value

Table 2 Effects of FMN concentration on dimer formation

FMN concn (mM)	Relative yield of dimers (relative value)
0 1	89
0 5	100
2 0	106
5 0	69

The reaction mixture contained 0 5 mM manganous chloride.

Values are expressed relative to the value at 0 5 mM

Table 3. Effects of $MnCl_2$ concentration on dimer formation

$MnCl_2$ concn (mM)	Relative yield of dimers (%)
0 1	64
1 0	100
10 0	102

The reaction mixture contained 0 5 mM FAD
Values are expressed relative to the value at 1 0 mM

(1–3 hr) leurosine and catharine were detected as well as vinamidine and 3-(*R*)-hydroxyvinamidine, but their amounts were relatively small and they disappeared with longer incubation times Formation of 3-(*R*)-hydroxyvinamidine, a major product, reached a maximum at 2 hr then gradually decreased while the amount of vinamidine, the other main product, continued increasing, even after 12 hr

Effects of pH and buffers

The effect of pH was studied using Tris–HCl and MES buffers (Fig. 3). The optimum pH for vinamidine and 3-(*R*)-hydroxyvinamidine formation was pH 6.8 MES buffer inhibited the formation of coupling products. Also, potassium phosphate and HEPES buffers showed inhibitory effects on coupling (data not shown)

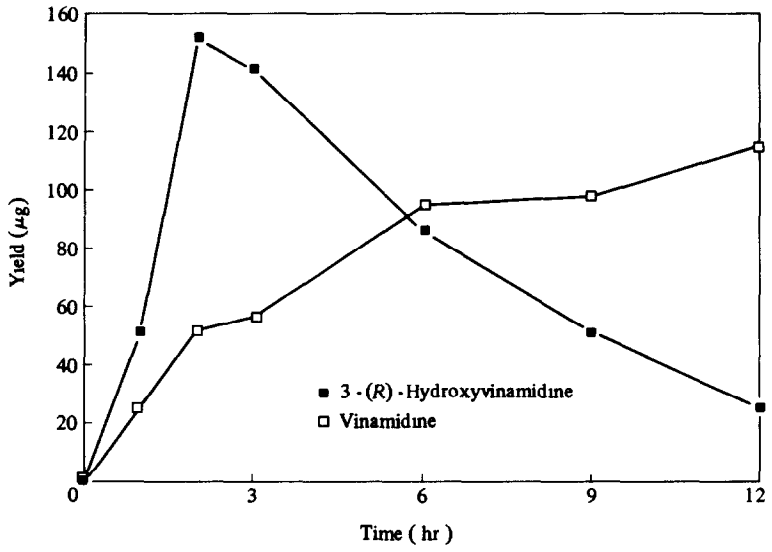

Fig 2 Time course of enzyme reaction The reaction mixture contained 1 mM FMN and 1 mM $MnCl_2$ in Tris–HCl buffer (100 mM, pH 6 8)

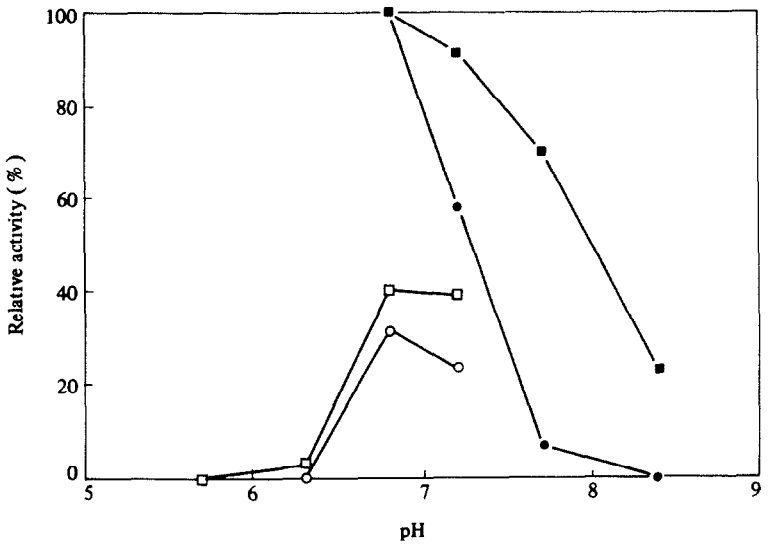

Fig 3 Effects of pH and buffers on dimer formation The reaction mixture contained 1 mM FMN and 1 mM $MnCl_2$ Values are expressed relative to those obtained at pH 6 8 in Tris–HCl ■ 3-(*R*)-Hydroxyvinamide. □ vinamidine

Non-enzymatic formation of dimeric alkaloids

Leurosine and catharine (and sometimes small amounts of vinamidine and 3-(*R*)-hydroxyvinamidine) were non-enzymatically formed upon incubation of vindoline and catharanthine with FMN (or FAD) and $MnCl_2$ (Table 4) The total conversion observed was comparable to that of the enzymatic reaction in the presence of $MnCl_2$

Isolation of 3',4'-anhydrovinblastine

In terms of vinblastine (VLB) and vincristine (VCR) synthesis, it would be preferable to produce 3',4'-anhydrovinblastine rather than its oxidized derivatives (leurosine, catharine and vinamidine) To prevent oxidation of AVLB, and as an attempt to convert at least some of the oxidized products to AVLB, mild reduction procedures were employed. Addition of an excess amount of $NaBH_4$ to the reaction mixture, after the reaction, resulted in the isolation of a substantial amount of AVLB. A detailed mode of action will be described elsewhere. The maximum yield of AVLB (*ca* 25%) was achieved after 1.5 hr incubation (Fig 4) Longer incubations resulted in a rapid decrease of AVLB, and oxidized derivatives of AVLB appeared. P7 (Fig 4) is an unidentified dimeric alkaloid which might be a reduced form of 3-(*R*)-hydroxyvinamidine formed by $NaBH_4$ treatment.

Although the presence of enzyme activity catalysing the coupling of vindoline and catharanthine to dimeric alkaloids has been demonstrated, this is the first report showing accumulation of a substantial yield of coupling products with an enzymatic system Isolation of AVLB in large amounts may open the possibility of economical production of the antitumour agents, vinblastine and vincristine through enzymatic methods.

At least two reaction steps are involved in the formation of oxidized derivatives of AVLB such as vinamidine and 3-(*R*)-hydroxyvinamidine First, vindoline and catharanthine are coupled to form AVLB, then the latter is oxidized From the results so far obtained, it is difficult to judge how many enzymes are involved in this overall pathway Involvement of a non-enzymatic step in the pathway must also be considered, as the evidence of non-enzymatic coupling was demonstrated (Table 3) Detailed mechanisms of the reaction should be clarified by

Table 4 Enzymatic and non-enzymatic formation of dimeric alkaloids

Enzyme	$MnCl_2$	Specific activity (nmol/mg protein/hr) Leu	Cat	VinOH	Vin	Total
+	+	0 44	0 57	14 26	1 87	17 14
+	–	0 73	0 58	–	–	1 31
Boiled	+	1 79	0 42	—	—	3 52
Boiled	–	0 38	0 27	—	—	0 65
–	+	2 11	0 80	–	–	2 91
–	–	0 54	0 24	—	–	0 78

Leu = Leurosine, Cat = catharine, VinOH = 3-(*R*)-hydroxyvinamidine, Vin = vinamidine, $MnCl_2$ concn = 1 mM

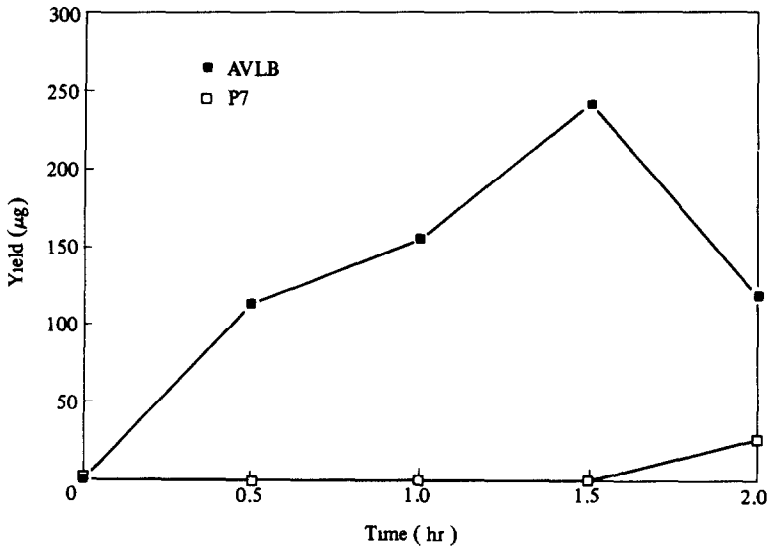

Fig 4. Time course of AVLB formation The reaction mixture consisted of 5 mg enzyme, 0.5 mg catharanthine HCl, 0.5 mg vindoline, 1 mM FMN, and 1 mM $MnCl_2$ in 6 ml Tris–HCl buffer (100 mM, pH 6 8).

studying both steps separately. The first step (i.e. formation of AVLB) will be discussed further in our next paper.

EXPERIMENTAL

Cell suspension cultures. A selected cell line, JWM*, was obtained from Dr W G. W. Kurz (Plant Biotechnology Institute, Saskatchewan, Canada) This suspension culture produced a large amount of catharanthine. The cells were maintained in Murashige and Skoog medium [5] with 1 mg/l NAA and 1 mg/l kinetin (60 ml medium in a 250 ml Erlenmeyer flask) on a rotary shaker (130 rpm) at 25° and subcultured every 2 weeks Two-week-old cells were harvested and stored at −20° until used.

Enzyme extraction Frozen cells were ground in a chilled mortar with two vols of Tris–HCl buffer (100 mM, pH 7) and an equivalent weight of PVPP. The ext was filtered through miracloth and centrifuged at 10,000 *g* for 20 min Protein was pptd from the supernatant with 70% satd $(NH_4)_2SO_4$ and obtained by centrifugation (10000 *g*, 20 min) The protein pellet was dissolved in Tris buffer and then desalted on a Biogel P-6 column equilibrated with the same buffer This desalted protein soln was used as the source of enzyme in the reaction All manipulations were performed at 0–4°.

Determination of protein concentration Protein concn was determined after ref [6]. Commercial horseradish peroxidase (Sigma) was used as standard

Reaction conditions The reaction mixt consisted of 5 mg extracted protein, 1 mg catharanthine HCl, 1 mg vindoline and cofactors in 6 ml Tris–HCl buffer (100 mM, pH 7) The reaction was carried out at 30° for 3 hr under static conditions and stopped by adding 28% NH_4OH (to pH 9)

Alkaloid extraction. Alkaloids were extracted from the reaction mixt with EtOAc × 3. The combined extracts were evapd to dryness, the residue dissolved in 0.5 ml MeOH and then subjected to Sephadex LH-20 gel filtration to separate dimeric products from the substrates. The dimeric fraction thus obtained was evapd to dryness and dissolved in an appropriate vol of MeOH for TLC and HPLC assay.

Product analysis by TLC and HPLC TLC solvent systems routinely used were (1) CH_2Cl_2–MeOH, 100 7 and (2) toluene–Me_2CO–MeOH–28% NH_4OH, 56 20:4 1 Developed silica gel plates (Baker, Si 250F) were scanned at 280 nm with a TLC scanner (Shimadzu, CS-930). HPLC was performed using a prog. gradient with H_2O–MeOH from 9:11 to 1 9 on a RP-8 analytical cartridge maintained at 30°. Alkaloids were detected at 226 nm with a 340 nm ref.

Identification of alkaloids Dimeric alkaloids formed were identified by 2-D TLC and colour reactions with ceric ammonium sulphate [7], R_f on HPLC, UV spectra and MS. The conversion yield was calculated by dividing the sum of the numbers of moles of each dimeric alkaloid formed by the number of moles of vindoline incubated

REFERENCES

1 Stuart. K. I, Kutney, J. P., Honda, T. and Worth, B R. (1978) *Heterocycles* **9**, 1419.
2. Scott, A I., Guerittte, F. and Lee, S. L (1978) *J. Am. Chem. Soc.* **100**, 6253.
3 Hassam, S B. and Hutchinson, C. R. (1978) *Tetrahedron Letters* **19**, 1681
4 Stuart, K K, Kutney, J. P., Honda, T. and Worth, B R (1978) *Heterocycles* **9**, 1391
5 Murashige, T and Skoog, F (1962) *Physiol Plantarum* **15**, 473
6 Bradford, M. (1976) *Anal. Biochem* **72**, 248
7. Kurz, W G W and Constabel, F. (1982) in *Plant Tissue Culture Methods* (Wetter, L. R and Constabel, F, eds), pp. 128–131 NRC, Saskatoon.