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CONVERSION OF ANHYDROVINBLASTINE TO VINBLASTINE BY CELL-FREE HOMOGENATES OF *CATHARANTHUS ROSEUS* CELL SUSPENSION CULTURES

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Abstract– A cultured cell-line of *C. roseus* (EU4A) which does not produce detectable amounts of vinblastine (**1**) or related *bis* indole alkaloids has been used to produce a cell-free homogenate which catalyses transformation [21′α-^{3}H] anhydrovinblastine (**5a**) into **1**.

Plant cell cultures represent a potential source of economically important secondary metabolic products.[1-4] Frequently however such cultures do not express the complete biosynthetic potential of the intact plant.[5, 6] In general the production of secondary metabolites by cultured plant cells falls into one of two patterns—either the final biosynthetic product is accumulated but in amounts far short of those observed for the intact plant or the final product is not produced in any quantity but instead its biosynthetic precursors are accumulated.[5]

The medicinal and economic importance of the *bis*-indole alkaloids, vinblastine (VLB, **1**) and vincristine (**2**) from *C. roseus* has prompted numerous studies of the biosynthetic capabilities of cultured cells of this plant over the past 20 yr.[8-15] While neither of the dimeric alkaloids (**1**) or (**2**) have been reported as biosynthetic products from *C. roseus* tissue culture

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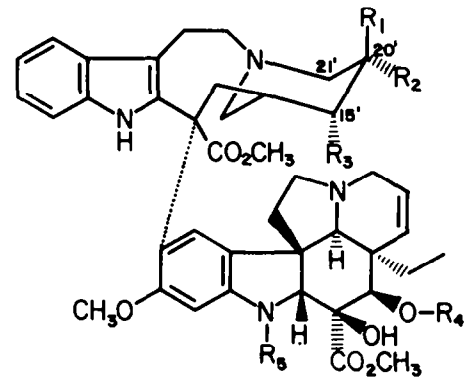


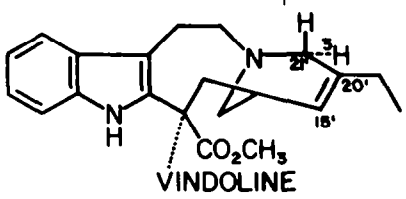
(1) Vinblastine R_1=OH; R_2=Et; R_3=H; R_4=Ac; R_5=CH_3

(2) Vincristine R_1=OH; R_2=Et; R_3=H; R_4=Ac; R_5=CHO

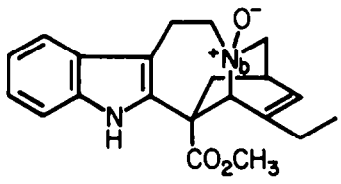
(3) Desacetylvinblastine R_1=OH; R_2=Et; R_3=H; R_4=H; R_5=CH_3

(4) Leurosine R_1=Et; R_2-R_3=O; R_4=Ac; R_5=CH_3

(5) Anhydrovinblastine R_1=Et; $\Delta^{15'(20')}$; R_3=H; R_4=Ac; R_5=CH_3



(5a)



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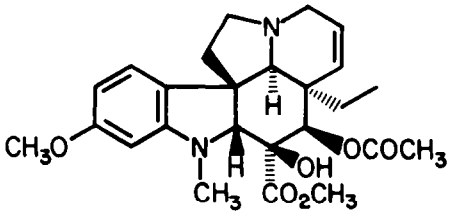

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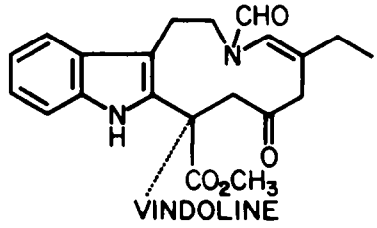

(8)

strains a number of monomeric alkaloids of both *Iboga* and *Aspidosperma* types have been isolated.[11-15]

In the course of a continuing study on indole alkaloid elaboration by *C. roseus* cultures we have investigated the capability of cell-free homogenates derived from cell cultures to catalyse the interconversion of intermediates in the VLB bio-synthesis pathway. We report here the conversion of anhydrovinblastine (*anhydro*-VLB, 5) to VLB by a cell-free homogenate derived from *C. roseus* (strain EU4A) suspension culture. *Anhydro*-VLB has previously been shown to be a precursor of VLB in *C. roseus* plants.[16-18]

RESULTS AND DISCUSSION

[21′α-^{3}H)Anhydro-VLB (**5a**) was prepared by the modified Polonofski coupling of catharanthine Nb oxide (**6**) and vindoline (**7**) and reduction of the resultant imine with sodium borotritiide.[16,19]

When radiolabelled *anhydro*-VLB was administered to suspension cultures of *C. roseus* virtually no uptake of the alkaloid into the cells could be detected. TLC radioscans of the dichloromethane extract of the medium 6 hr after administration showed that almost all of the added alkaloid had been degraded to materials the principal of which coeluted with leurosine (**4**) and catharine (**8**). Oxidation of anhydro-VLB to leurosine (**4**) in aereated solvents has been described previously.[20] Faced with the fact that the rate of degradation of the precursor greatly exceeded uptake by the cells it was necessary to examine the possible conversion in cell-free extracts where transport of *anhydro*-VLB would not be a rate determining factor.

Accordingly [21′α-^{3}H]*anhydro*-VLB (**5a**) was fed to a cell-free homogenate of suspension culture cells *C. roseus*, (EU4A) prepared in a similar manner to that used for leaf-tissue.[21] At the end of a 3 hr incubation vinblastine sulphate was added as carrier and the VLB reisolated and rigorously purified by successive TLC, reversed phase HPLC and crystallisation to constant specific activity. The results of two independent experiments carried out with different batches of cells and independent preparations of [21′α-^{3}H] *anhydro*-VLB are shown in Table 1. No incorporation of activity was detected when the homogenates were boiled for 15 min prior to incubation showing that no non-enzymic hydration of the 15′20′ double bond of **5** occurs.

Methanolysis of the isloted VLB afforded the desacetyl derivative **3** which was purified by TLC and subsequently reacetylated with AC_2O-pyridine to give

Table 1.

Expt	[21′-^{3}H](5) administered		VLB (1) isolated[a]		Incorporation %
	dpm	dpm/mg	dpm[b]	dpm/mg	
1	6.60×10^6	1.12×10^7	3.79×10^4	4062[d]	0.57
			0.98×10^3	105[d]	0.02
2[c]	6.21×10^6				
3	2.91×10^7	1.34×10^8	1.41×10^5	6060[e]	0.49
			3.73×10^3	160[e]	0.01
4[c]	2.92×10^7				

(a) as ½MeOH ½Et_2O solvate; (b) mean of constant activity samples each counted to a 2σ% error of <3; (c) extracts boiled for 15 minutes prior to incubation; (d) 10 mg of VLB H_2SO_4 added as carrier; (e) 25 mg of VLB H_2SO_4 as carrier.

VLB which showed no loss of activity, thus precluding a possible exchange of label *via* chemical degradation of **5a** and reincorporation of tritium into the acetyl moiety. Exchange of the acetyl group of *anhydro*-VLB in homogenates from leaf-tissue had been shown to occur in earlier studies when [21′-^{3}H,*acetyl*-^{14}C]*anhydro*-VLB was employed as a substrate.[22] Enzymic deacetylation of vindoline (**7**) by suspension cultures of *C. roseus* has recently been reported.[23]

The above results clearly show that the enzymatic activity responsible for the formal hydration of *anhydro*-VLB to VLB is present in this strain of *C. roseus* cell culture despite the fact that no dimeric alkaloids are produced in detectable amounts by cell cultures. At this stage it is not known whether the pathway to vinblastine is blocked at an earlier stage by the absence of specific enzymes or whether the total synthetic capability is present but repressed in some manner. The latter possibility would be consistent with the results of earlier studies on the formation of terpenoids and tropane alkaloids in other plant cell cultures.[7]

EXPERIMENTAL

General. M.ps were recorded on a Kofler hot-stage apparatus and are uncorrected. TLC was performed on 0.25 mm layers of silica gel (Merck GF_{254}). Solvents were dried and purified by standard procedures and organic extracts dried with Na_2SO_4. HPLC was carried out using a Waters chromatograph and eluted alkaloids detected by their absorbance at 254 nm. Radioactivity was measured in Beckman Ready-Solv-NA scintillation cocktail on a Beckmann LS-700 Scintillation Counter. TLC-radioscans were carried out on a Panax radioscanner.

Growth of cell cultures and preparation of cell-free extracts. The *C. roseus* tissue culture cell line used in this study (EU4A) was selected from cell-line CRW[11] which has been subcultured in our laboratory since 1980. Suspension cultures were grown in Shenk and Hildebrant medium[24] (100 ml in a 250 ml Erlenmyer flask) under constant illumination at 27 ± 0.5° on an orbital shaker at 120 rev/min. For preparation of cell-free extracts cells were harvested at early stationary phase (14 d from an inoculation volume of 10%) yielding 40% packed cell volume (22 g/100 ml culture fresh tissue). The cell-free extracts were prepared essentially as described by Scott and Lee[20]. Fresh tissue (50 g) was homogenised in tris maleate buffer (50 mM, 100 ml, pH 7.0) for 3 min at high speed in a Waring blender, centrifuged at 35,000 g to remove cell debris and the supernatant stored at 5° until required for incubation.

Preparation of [21′α-^{3}H]*anhydrovinblastine* (**5a**).[19] Catharanthine Nb oxide (47 mg), prepared as previously described,[10] and vindoline (63 mg) in dry CH_2Cl_2 (400 μl) at −78° under Argon were treated with trifluoracetic anhydride (55 μl) with continual stirring. After 0.5 hr the soln was evaporated *in vacuo* at 10°. The residue was dissolved in MeOH (1.5 ml) cooled to 5° and treated with a methanolic soln (0.2 ml) of sodium borotritiide (Amersham, 25 mCi, 50 Ci/mmol). After 5 min a methanolic soln (1 ml) of $NaBH_4$ (20 mg) was added and the soln allowed to come to room temp over a period of 0.5 hr. The mixture was poured into water (10 ml) extracted with CH_2Cl_2 (10 ml × 3), the dried organic extract evaporated *in vacuo* and the components separated by TLC on silica gel using 5% MeOH-CH_2Cl_2 (× 2) as eluant. Subsequent crystallisation from MeOH afforded **5a** (35 mg, 1.34×10^8 dpm/mg) m.p. 207–210° (lit.[19] 208–210°).

Incubation of [21′α-^{3}H]*anhydro VLB* (**5a**) *with cell-free extracts.* Typically aliquots (200 μl) of an ethanolic soln (2 ml) of **5a** were added to 50 ml of the cell-free extract prepared as above and incubated with shaking at 29° for 3 hr. For controls (expts 2 and 4) the extract was boiled for 15 min and cooled prior to addition of **5a**. After incubation vinblastine sulphate hydrate (10 mg) was added as carrier, the extract adjusted to pH 10 by addition of 3M NH_4OH, extracted with CH_2CL_2 (25 ml × 3) and the dried extract evaporated *in vacuo*. Vinblastine was reisolated by preparative TLC of the residue (12 mg) on silica gel using 10% MeOH-CH_2Cl_2 as eluant and purified by preparative HPLC on a Waters μBondapak C-18 column (25 × 0.2 mm) eluted with 50% MeCN aq. phosphate buffer (0.01 M, pH 7.2), flow rate 2.5 ml/min, back pressure 180 kg/cm^2. Crystallisation from MeOH–ether afforded vinblastine as its MeOH–ether solvate m.p. 206–211°. Subsequent recrystallisation afforded material of constant specific activity (Table 1).

Deacetylation of [^{3}H]*-vinblastine.* Vinblastine from the above experiment (expt 3, Table) was further diluted (total 17 mg, 755 dpm/mg) and dissolved in 0.1 M NaOMe–MeOH (0.5 ml). The soln left at room temp overnight and then poured into water (3 ml). Extraction with CH_2Cl_2 (5 ml × 2), evaporation and preparative TLC on silica using 10% MeOH–CH_2Cl_2 as eluant afforded *desacetylvinblastine* (**3**, 8 mg).[25] Treatment of **3** with Ac_2O (50 μl) in pyridine (100 μl) overnight followed by evaporation of the reagents *in vacuo*, preparative TLC of the product and crystallisation from MeOH–ether gave vinblastine (5 mg, 726 dpm/mg), the specific activity of which was unchanged by repeated recrystallisation.

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