ALKALOID PRODUCTION IN CATHARANTHUS ROSEUS (L.) G. DON CELL CULTURES. XIV.[1] THE ROLE OF UNSTABLE DIHYDROPYRIDINIUM INTERMEDIATES IN THE BIOSYNTHESIS OF BISINDOLE ALKALOIDS

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Abstract - Employing enzyme systems obtained from Catharanthus roseus cell cultures, experiments relating to the biosynthesis of various bisindole alkaloids are described. Specifically, the enzyme-catalyzed coupling of catharanthine (1) and vindoline (2) to a highly unstable dihydropyridinium intermediate (9), important in the biosynthesis of 3',4'-anhydrovinblastine (3), leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8) is presented. Conversions of 9 to these alkaloids and its role in the biosynthesis is discussed. Although catharanthine is well utilized by the enzyme system, catharanthine N-oxide (10), an important intermediate in the chemical coupling of 1 and 2 to afford 9, is not utilized.

Our earlier studies with cell-free enzyme systems from Catharanthus roseus plants[2,3] showed that 3'4'-anhydrovinblastine (3) can be obtained in an enzyme-catalyzed coupling of the monomeric alkaloids catharanthine (1) and vindoline (2), and that 3, under appropriate conditions, can be converted into the naturally occurring bisindole alkaloids leurosine (4), vinblastine (5) and catharine (6) (see Scheme 3). It was implied[2] that the highly unstable dihydropyridinium intermediate (9) could be important in terms of its role in linking the biosynthetic pathways of 4, 5 and 6 with the starting alkaloids (1 and 2). It was clear that a 1,2-reduction of 9 by, for example, a NADPH-type enzyme system present in the cell free systems obtained from C. roseus plants would afford 3. Although 3 could be a product of this type of enzymatic reduction it would not necessarily follow that 3 is a mandatory precursor on the

biosynthetic pathway leading to 4, 5 and 6. Evidence for the direct involvement of 9 and/or 3 was difficult to establish in studies involving plant systems but with plant tissue cultures which provide a more easily controlled system, important information relating to this aspect should be attainable. A recent review[4] summarizes briefly some of our studies in this direction but it is now appropriate to discuss these studies in more detail and, in particular, to present our most recent results which shed important light on this aspect of biosynthesis of bisindole alkaloids.

The first important objective of this study was to develop methodology for the actual detection of the highly unstable dihydropyridinium intermediate 9 in order to study its eventual appearance, if any, in the enzyme catalyzed coupling of 1 and 2. For this purpose, the previously developed[5,6] method of coupling catharanthine via its N-oxide intermediate (10) with vindoline (2) to afford 3 (Scheme 1) was investigated. It is clear from the mechanism involved in this reaction that the intermediated formed must possess the dihydropyridinium structure 9. Indeed when 2 and 10 are coupled in the presence of trifluoroacetic anhydride at -60°C in an argon atmosphere, the volatile reagents carefully removed in vacuo at low temperature (-20°C), the intermediate 9 can be trapped and analyzed by HPLC (H_2O:MeOH, 23:77 containing 0.1% triethylamine as modifier, reverse phase C-18 column, retention time, 8 minutes with flow rate, 1.5 ml/min). With degassed solvents such as methanol, methylene chloride, acetonitrile, dimethylformamide and tetrahydrofuran, the intermediate 9 could be analyzed even at room temperature. With data on 9 on hand, the enzymatic coupling of 1 and 2 could now be properly evaluated in terms of rate of formation, if any, of 9, its bioconversion to other alkaloids, etc.

Employing a stable cell line (coded as AC3), various incubations with catharanthine and vindoline were performed. Various preparations (crude cell free extract, supernatant fraction, pellet fraction) as shown in Scheme 2, were utilized. Tables I and II summarize the data obtained.

First of all, it was established that when 1 and 2 were incubated with the preparations shown in Tables I and II, the first intermediate formed in short time periods (5-10 minutes) was identical with the chemically synthesized 9. Rapid biotransformation of 9 into the various bisindole alkaloids 4, 6, 7 and 8 then occurred. Clearly a multi-enzyme complex, still present in our various preparations, was rapidly converting 9 into the higher oxidation products leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8). Vinblastine (5)

was not observed in these experiments so that different enzymic systems are obviously involved in the transformation of 9 to 5.

Age of culture is an important factor. Thus young cultures (5 days old) in early log growth stage had the highest coupling activity yielding 18% of bisindole alkaloids after a 5 hr incubation in the presence of FAD and $MnCl_2$ as cofactors (Table I). Other cofactors such as

Scheme 1. Mechanism for Coupling of Catharanthine and Vindoline. Chemical versus Biological Route.

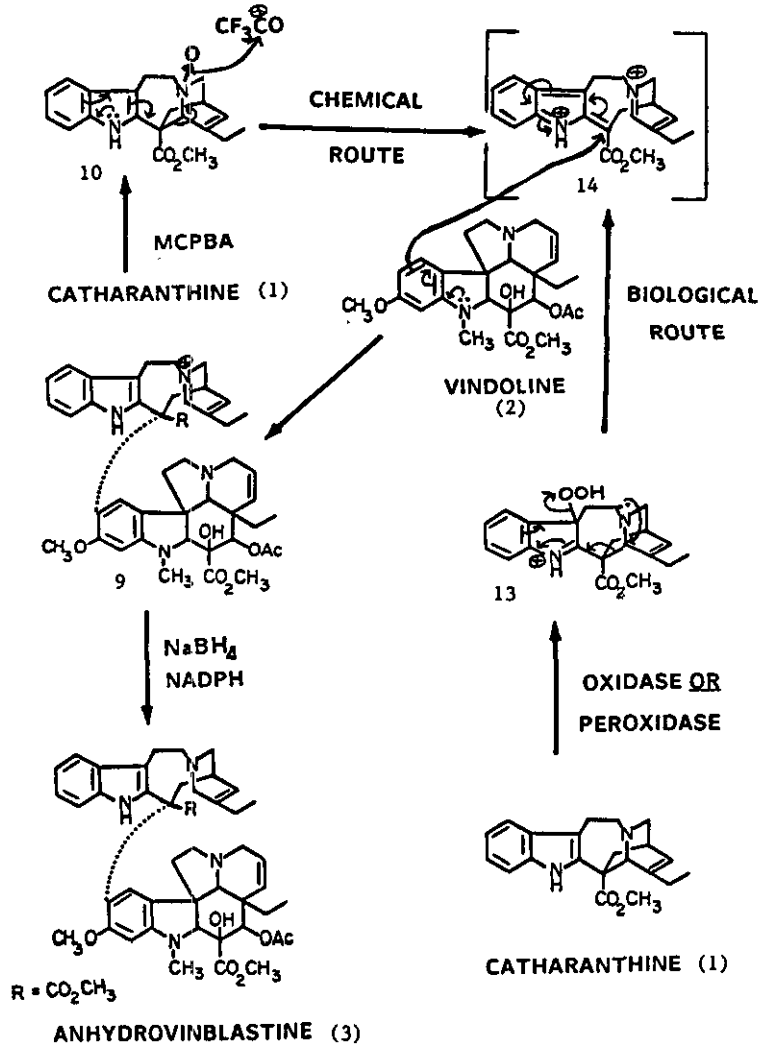


Table I. Coupling of Catharanthine (1) and Vindoline (2) Employing Crude Enzyme (C.F.E.) Derived from Varying Ages of C. roseus Cell Cultures (AC3)[a]

Age of Culture (days)	C.F.E.[b] Protein conc. (mg/ml)	Wt. of total Bisindole alkaloids (mg)[e]	Yield of Coupling (%)	Bisindole alkaloids identified (Relative % of total dimers)[f]			
				4	6	7	8
5	crude[c] (0.75)	12.5	18	1.9	27.4	7.2	1.4
5	u-C.F.E.[d] (0.54)	3.2	4.6	7.8	29.7	8.1	2.5
9	crude[c] (0.9)	9.9	14.2	2.1	22.4	7.2	1.4
9	u-C.F.E.[d] (0.65)	1.8	2.6	3.0	11.7	2.2	0.6
12	crude[c] (0.96)	3.9	5.6	1.3	8.7	-	4.9
12	u-C.F.E.[d] (0.82)	2.3	3.3	2.2	10.7	1.3	-

a Incubation conditions: catharanthine (40 mg), vindoline (40 mg), FAD (44 mg) $MnCl_2$ (6.8 mg), C.F.E. (120 ml); incubated at 26° C for 5 h.

b C.F.E. prepared in 0.1 M phosphate buffer (pH=6.3) (Scheme 2)

c Crude C.F.E. referred to the supernatant fraction at 20,000g (20 min.) (Scheme 2)

d u-C.F.E. referred to the supernatant fraction at 150,000g (2 h)(Fraction S, Scheme 2)

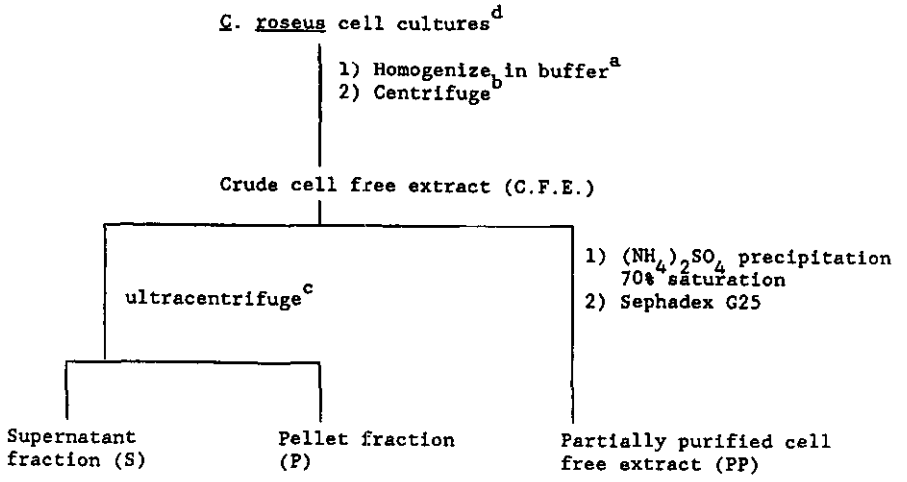
e Isolated by Sephadex LH20 column chromatography.

f As determined by HPLC analysis

NADP and cobalt ion ($CoCl_2$), as well as reductants such as NADPH, β-mercaptoethanol, sodium azide and dithiothreitol (DTT) were also evaluated. The reductants, in general, inhibited completely the coupling of 1 and 2 and neither 9 nor any of the bisindole compounds 3, 4, 6, 7 and 8, were observed in the reaction medium even after longer incubation periods.

Table II provides a comparison of the crude cell free extract (C.F.E., Scheme 2) and the partially purified enzyme fraction (PP, Scheme 2) in terms of enzymatic coupling activity of 1 and 2 to afford 9 and, in turn, the bisindole alkaloids, leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8). A decrease by 45% in the yield of bisindole alkaloids (compare 5.6% versus 10.2% in Table 2) was observed thereby revealing considerable denaturation of the protein during attempted purification.

Scheme 2. Procedure for Preparation of Crude Cell Free Extract, Supernatant Fraction, Pellet Fraction and Partially Purified Cell Free Extract.

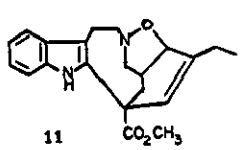


a) Homogenization in potassium phosphate buffer (0.1 M, pH 6.3) or Tris-HCl buffer (0.1M, pH 7.5) in the presence of an equal weight of Polyclar AT.

b) Centrifugation at 20,000 - 25,000 g for 20 min.

c) Ultracentrifugation at 150,000 g for 2 h.

d) All processes were conducted at 4°C or lower.



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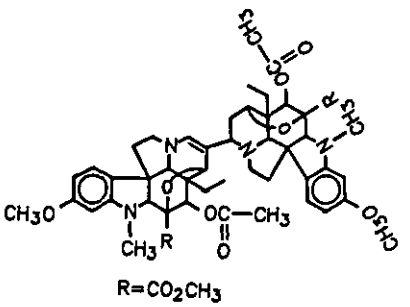


R=CO_2CH_3

12

Table II. Coupling of Catharanthine (1) and Vindoline (2) by Crude and Partially Purified (P.P.) Enzymes (C.F.E.) of C. roseus Cell Cultures (AC3 line)[a]

C.F.E.[b] (ml)	Wt. of Catharanthine (mg)	Wt. of Vindoline (mg)	Wt. of FAD (mg)	Wt. of $MnCl_2$ (mg)	Wt. of total bisindole alkaloids (mg)[e]	Yield of coupling (%)	Bisindole alkaloids identified (Relative % of total dimers)[f]			
							4	6	7	8
crude[c] (80 ml)	27	27	60	4.6	5.3	10.2	1	12.1	2.9	0.9
P.P.[d] (90 ml)	30	30	67	5	2.9	5.6	4.3	15.3	7.7	2.5

[a] Age of culture = 10 days in 1B5 medium.

[b] C.F.E. prepared in 0.1M tris -HCl buffer (pH=7.5). (Scheme 2) Incubation time = 5 hrs at 26°C

[c] Protein concentration = 0.99 mg/ml

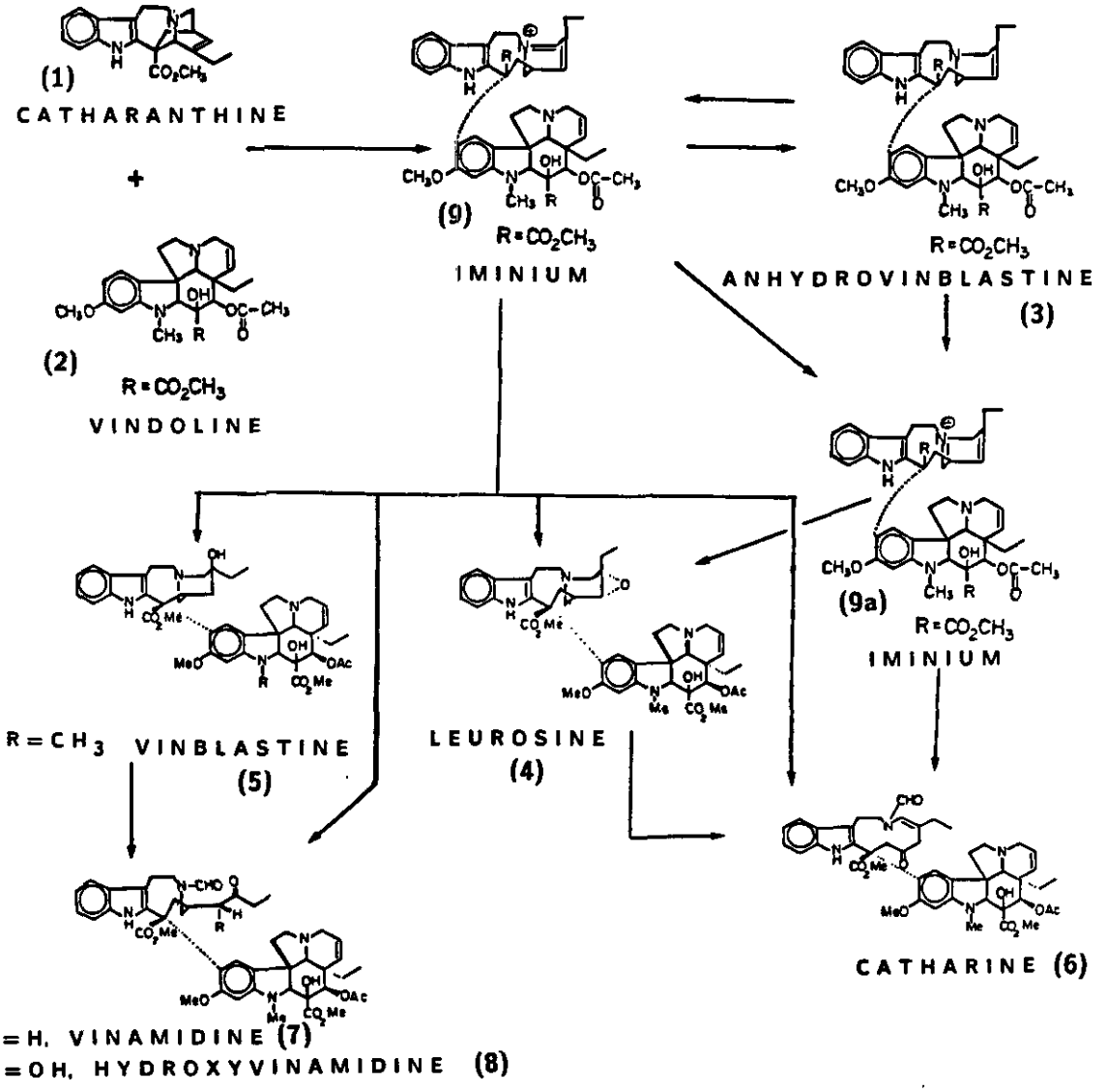
[d] Protein concentration = 1.05 mg/ml

[e] Isolated by Sephadex LH20 column chromatography

[f] As determined by HPLC analysis

Since we have established that the same conjugated dihydropyridinium intermediate 9 is involved in both the chemical coupling of catharanthine N-oxide (10) with vindoline (2) (Scheme 1) and in the enzymatic coupling of 1 and 2, it was of interest to determine whether the N-oxide intermediate may also be involved in the enzymatic process. The N-oxide was prepared and since it is known to be somewhat unstable and undergoes a thermal [2,3]-sigmatropic rearrangement to an oxazolidine (11)[7], its stability in the various buffer systems containing the cofactors (Table 1) was evaluated. Under the usual incubation conditions, 10 was found to be stable, and therefore 10 and 2 were utilized as substrates in the studies under conditions summarized in Table 1. HPLC analyses revealed very little biotransformation of the monomeric substrates and neither 9 nor any of the bisindole products noted above were observed. A small quantity (ca 2%) of a product previously isolated as an oxidative metabolite of vindoline and assigned the structure 12[8] was obtained. These results indicated that the N-oxide is not an intermediate and that a "biological" mechanism somewhat different from that of the chemical coupling is involved. An attractive alternative mechanism is proposed in Scheme 1. A hydroperoxyindolenine intermediate 13, suggested in one of our earlier[2] publications and

Scheme 3. Overall Summary of Enzymatic Coupling of Catharanthine (1) and Vindoline (2) to Bisindole Alkaloids.



readily derived by well-known[9] enzymatic and biomimetic reactions of indole systems can in fact, provide the same highly reactive intermediate 14 for spontaneous coupling with the nucleophilic vindoline unit. The resultant product is the earlier established intermediate 9. A comparison of chemical versus biological routes to 9 is provided in Scheme 1.

In conclusion, our studies have conclusively established the role of 9 and its fate in enzyme-

catalyzed conversions to various bisindole alkaloids within the vinblastine family of compounds. A better understanding of the biosynthetic pathway is on hand. Clearly the present study reveals that the more stable oxidative enzymes, responsible for conversions of 9 to the higher oxidized products 4, 6, 7 and 8, predominate during enzyme isolation and attempted purification. Finally, improvements in enzymatic yields of bisindole compounds from 1 and 2, via 9, are highly desirable and such studies are presented in the accompanying publication.

ACKNOWLEDGEMENTS

Financial aid from the Natural Sciences and Engineering Research Council of Canada and the National Research Council of Canada (PILP Program) is gratefully acknowledged.

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Received, 4th November, 1987