ALKALOID PRODUCTION IN CATHARANTHUS ROSEUS (L.) G. DON CELL CULTURES. XVI[1]. BIOTRANSFORMATION OF 3',4'-ANHYDROVINBLASTINE WITH CATHARANTHUS ROSEUS CELL CULTURES AND ENZYME SYSTEMS

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Abstract - Employing cell cultures of Catharanthus roseus and enzyme systems derived from such cultures, a detailed study of the biotransformation of 3',4'-anhydrovinblastine (3) is described. It is shown that enzymatic conversion of 3 to the isolated end products, the bisindole alkaloids leurosine (4), vinblastine (5), catharine (6), vinamidine (7) and hydroxyvinamidine (8) proceeds initially to the same dihydropyridinium intermediate (9) obtained when the monomeric alkaloids catharanthine (1) and vindoline (2) are enzymatically coupled. These data establish that the overall biosynthetic pathway to the bisindole alkaloids proceeds from 1 and 2 to the initially formed 9 and the latter, under enzymatically controlled oxidation-reduction, affords the alkaloids 3-8. However, when 3 is incubated as substrate, the enzymes are capable of utilizing and converting it into the alkaloids 4-8. In summary, 3 is not a direct biosynthetic precursor of 4-8.

In the previous accompanying publications[1,2], we described our studies with cell-free enzyme and immobilized enzyme systems derived from Catharanthus roseus cell cultures and the ability of such systems to couple catharanthine (1) and vindoline (2) to afford various bisindole alkaloids. One of the alkaloids, 3',4'-anhydrovinblastine (3)(Scheme 1) was formed as a major product especially when the enzyme incubation mixture was reduced with $NaBH_4$ just prior to

workup. It was of interest and importance to determine if 3 was indeed a biosynthetic precursor to the various other bisindole alkaloids obtained, for example, leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8) (Scheme 1). We[3,4,7,8] and others[5,6,9] had shown earlier that if 3 is administered to cell-free extracts derived from C. roseus plants[3-6] and cell cultures[7-9], it is converted to leurosine (4), catharine (6) and vinblastine (5). These latter studies merely indicated that 3 could be utilized as a substrate in enzymatic transformation to these compounds but clearly did not establish its precursor role if any. With our recent studies and particularly with the recognition of the dihydropyridinium intermediate (9) as the first-formed bio-intermediate in the enzymatic coupling of 1 and 2, the role, if any, of 3 becomes an open question. For this reason, further studies concerning the biotransformation of 3 with our more highly developed cell lines and/or enzyme systems became highly desirable.

Initial experiments with C. roseus cell cultures, that is, whole cells, were performed to obtain data on the biotransformation of 3. Such parameters as age of culture versus enzymatic activity to biotransform 3 were evaluated. It was shown that younger cultures (5-10 days old) showed a lower enzymatic activity in biotransforming 3 than did older cultures (13-25 days old). In these experiments, in addition to leurosine (4), vinblastine (5) and catharine (6) obtained in our earlier studies with enzymes from C. roseus plants[3,4] and cell cultures [7,8], two additional metabolites, vinamidine (7) and hydroxyvinamidine (8) were observed. Indeed the latter two compounds were often the major metabolic products, thereby revealing a high level of "oxidase and/or peroxidase" activity in our cultures.

In order to determine whether the enzymes responsible for these biotransformations are present within the cells and remain there during the entire culture period (5-30 days in the nutrient medium), a series of "spent" medium experiments were carried out. Filtration of the cell material after a given growth period provided the "spent" medium which was simultaneously evaluated with the whole cell culture (cells and medium) for their activity to biotransform 3. Enzymatic activity in the "spent" medium is minimal when a short growth period, for example, 5 days is involved. On the other hand, older cultures, for example, 18-30 days old, which showed high degrees of cell lysis, under microscopic observation, provided "spent" medium samples with significant enzymatic activity. Table I summarizes our results with the AC3 line in which cells in their early stationary phase (18 days old) were utilized. Parallel whole cells and "spent" medium incubations with 3 were carried out simultaneously. In both cases, the substrate 3 was completely converted after 15 h. Analyses of the metabolites obtained

Table I. Biotransformation of 3',4'-anhydrovinblastine (3) by C. roseus cultures (cell line AC3) in 1-B5 medium.[a]

Biotransformation medium	Incubation time (h)	Wt. of substrate 3 (mg)[b]	Wt. of basic cell extract (mg)	Wt. of basic supernatant extract (mg)	Recovered 3, (%)	Metabolites identified and % Yield
Whole cells	15	160	182.2	145	Trace < 1	5, 1.1% 4, 8.3% 6, 13.3% 7, 12.0% 8, 1.4%
"Spent" medium	15	140	-	140	0	4, 2% 6, 4.7% 7, 3.6% 8, 23%

[a] Age of culture = 18 days

[b] Concentration of substrate = 100 mg/l, added as hydrogensulfate salt

showed some differences in the ratio of the major products. In the whole cell experiment, catharine (6) (13.3%) and vinamidine (7) (12%) were the main metabolites, while hydroxyvinamidine (8) (23%) was the major product obtained on incubation with the "spent" medium. Of particular interest was the isolation of vinblastine (5) (1.1%) in the whole cell experiment but not in the "spent" medium experiment. This result suggested that the desirable enzyme system(s) for the transformation of 3 to 5 are associated with the cells that are in early stationary phase and that this enzyme system(s) may not be stable once released into the culture medium. Furthermore, enzymatic processes which occurred within the cells could have the benefits of secondary metabolites synthesis and storage stabilized by compartmentation[10] to minimize catabolism and degradation. The enzyme mixtures present in the "spent" medium do not provide such protection, so that the final metabolites obtained represent the overall biotransformations by the predominant enzymes present.

With the above data on hand, our attention turned to studies involving biotransformation of 3 with enzyme systems derived from C. roseus cultures utilizing the purification procedure outlined in the accompanying publication[2] (see Scheme 2). Employing HPLC methods described earlier[2] it was possible to monitor carefully the course of biotransformation of 3 and formation of metabolites at reaction time intervals in time course studies.

Table II. Biotransformation of 3 by cell-free enzymic systems from C. roseus cell cultures (cell line AC3)[1].

Age of Culture (days)	Type of cell-free extract[2]	% Conversion of 3 after 6 h incubation	% of 9 after 6 h incubation	Max. level of 9 (%)	Incubation time in hrs required to *reach max.* level of 9
5	C	85	41	53	3, (64)[3]
5	S	75	35	43	8, (82)[3]
5	P	43	22	30	7, (53)[3]
12	C	63	53	53	6, (63)[3]
12	S	60	47	47	6, (60)[3]
12	P	85	14	30	1, (47)[3]

[1] Cell-free extracts and biotransformations were carried out in phosphate buffer (0.1 M, pH 6.3). FAD (1 eq.) was added and experiments conducted at 26° C.

[2] C = crude cell-free extract; S = supernatant fraction after ultracentrifugation at 150,000 g; P = pellet fraction after ultracentrifugation at 150,000 g (Procedure is Scheme 2[2])

[3] The number in parenthesis refers to the % conversion of 3 at the time when maximum level of 7a was observed.

Table III. Biotransformation of 3 by cell-free enzymic systems from C. roseus cell cultures (cell line AC3)[1]

Type of Cell-free Extract	$MnCl_2$ (1 eq)	% Conversion of 3 after 6 hour incubation	% of 9 after 6 h incubation	Max. level of 9 (%)	Incubation time in hrs required to reach max. level of 9	% of 3 remaining at end of incubation (13 h)	% of 9 at end of incubation (13 h)	Metabolites identified (%) 5	4	6	7	8
crude	-	75	37	58	4, (64)[3]	0	7	1	3.1	10.5	6.6	4.2
crude	+	84	30	58	4, (68)[3]	0	12	1	2.3	6.8	6.2	6.2
PP[2]	-	68	21	28	8, (78)[3]	15	12	-	6.1	10.2	8.7	1.1
PP[2]	+	70	32	38	2, (57)[3]	6	7	-	0.8	4.2	6.2	4

[1] Cell-free extracts and biotransformations carried out in Tris-HCl buffer (0.1 M, pH 7.5). FAD (1 eq.) was added and experiments conducted at 26° C.

[2] PP = partially purified cell-free extract. Procedure is Scheme 2[2] except that Tris-HCl buffer was employed.

[3] The number is parenthesis refers to the % conversion of 3 at the time when maximum level of 9 was observed.

In order to obtain information related to enzyme stability within the cell system and to establish differences in enzymatic activity in various ages of cultures, we performed experiments with a number of suspension cultures grown in bioreactors (up to 30 ℓ size). These cultures were harvested at different times around the transition period between late log growth stage and early stationary phase (10, 12 and 14 days old), frozen and stored at -20° C for various periods of time (up to one year). These frozen cultures were then used to prepare the corresponding cell-free enzymic extracts (Scheme 2)[2] and analysed for activity to transform 3. Employing the direct HPLC monitoring techniques, it was found in all instances that complete disappearance of 3 occurred after an incubation time of 2 to 6 h with the above cell-free enzymic extracts and containing 1 equivalent FAD at 26-30° C. When these incubations were conducted at 0° C, rate of biotransformation decreased threefold and conversion of 3 required more than 6 h.

Careful HPLC monitoring of the above incubations revealed that the first bio-intermediate in the biotransformation of 3 is identical with the dihydropyridinium intermediate 9 synthesized unambiguously via the Polonovski reaction[2] (Scheme 1). In summary, the enzymatic coupling of catharanthine (1) and vindoline (2), as shown in the accompanying publications[1,2], and enzymatic biotransformation of 3 provide the same initial biointermediate 9.

Having established the formation of 9 in the initial step in the biotransformation of 3, it was important to understand the rate of formation of 9, its accumulation, if any, and the rate of its conversion to the various bisindole alkaloids. Tables II and III summarize a detailed investigation which addresses these issues. From Table II, it is clear that the highest activity to biotransform 3 is seen in the crude cell free extract from a 5 day old culture with accumulation of the biointermediate 9 reaching a maximum level (53%) after 3 h. HPLC analyses of the products obtained showed leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8).

In the next series of experiments (Table III), the effect of using a slightly basic buffer (Tris-HCl, 0.1 M, pH 7.5) in the preparation of the cell free extract (Scheme 2[2]) on biotransformation activity was evaluated. Further, purification by precipitation with ammonium sulfate (70%), desalting on Sephadex G25 to give a partially purified (PP) sample and the effect of manganese ions was established. In summary, little difference in biotransformation activity is shown in phosphate (pH 6.3) and Tris-HCl buffers while Mn^{+2} ion increases biotransformation of 3 (75% versus 84% in 6 h). The level of formation of 9 appeared similar in both studies. The partially purified (PP) system has a lower enzymatic activity. HPLC

analyses revealed the presence of the above-noted bisindole products. Of special interest is the presence of vinblastine (5) in the biotransformation of 3 with the crude cell free extract but not with the PP cell free extract. This supports the deduction mentioned earlier that the enzymic systems responsible for transforming 3 to 5 are quite unstable, so that deactivation can readily occur on further processing. The other metabolites identified included leurosine (4), catharine (6), vinamidine (7) and hydroxyvinamidine (8), similar to those obtained in above-mentioned cell and "spent medium" biotransformation of 3.

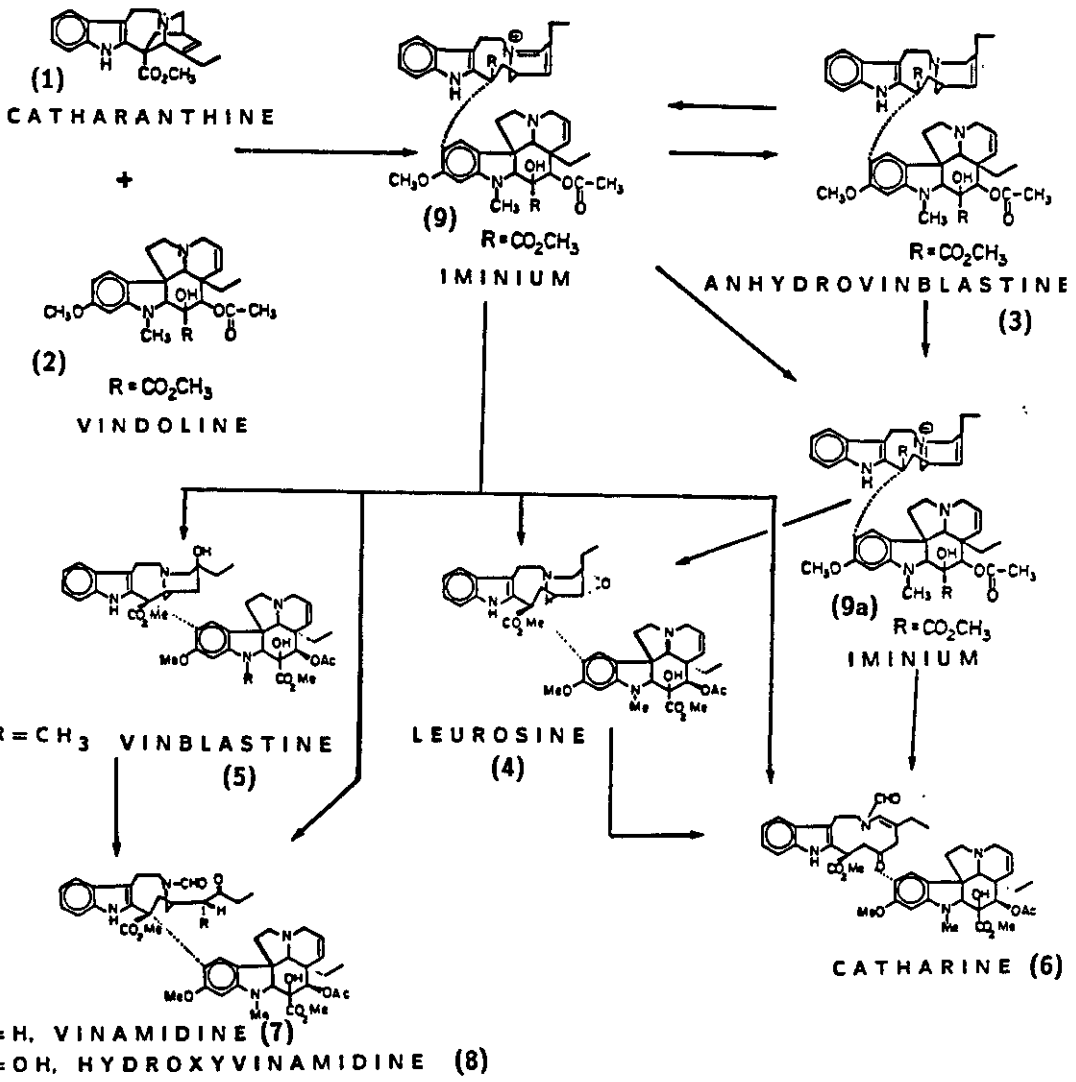
The low yield of vinblastine (5) obtained in the above studies, thus far, may be rationalized in several ways. These are: a) higher instability of the required enzymes to elaborate 9 to 5 and therefore destruction of enzymic activity during the isolation processes as summarized in Scheme 2[2]; b) rapid conversion of 5, once formed, by the predominant oxidases present, to higher oxidation products. Indeed some information pertaining to (b) is on hand from our recent studies. When vinblastine was incubated with a crude cell-free extract (Scheme 2)[2] with FAD (1 equivalent) as co-factor at 26° C, direct HPLC monitoring revealed a rapid biotransformation (30% in 30 minutes and complete conversion in 3 h) to other products. Subsequent separation of the reaction mixture indicated >50% yield of vinamidine (7). Hydroxyvinamidine (8) was not observed so its production, as noted in the above experiments, must derive from iminium 9 via other undetermined intermediates. In summary, the low yield of 5 is due, at least in part, to this rapid oxidative conversion to 7.

In conclusion, the above studies clearly demonstrate that 3',4'-anhydrovinblastine (3) is not the true biosynthetic precursor of the bisindole alkaloids. It is rather one of the products formed by 1, 2-reduction of the dihydropyridinium intermediate 9 and if incubated with an appropriate enzyme system merely reverts to 9 and the latter then undergoes biotransformation to the bisindole alkaloids 4-8. Therefore the intermediate 9 is the real biosynthetic precursor when the starting alkaloids catharanthine (1) and vindoline (2) are employed as the biosynthetic building units.

It should be noted that in Scheme 1 in this publication as well as in the accompanying publications[1,2], another iminium intermediate 9a is proposed in attempting to rationalize one possible pathway from 3 and/or 9 to the alkaloid catharine (6). We have no direct evidence for the existence of 9a in any of our incubation experiments.

Finaly, the results of the earlier[1,2] and this publication add important information to the overall understanding of the biosynthesis of this family of bisindole alkaloids. It is clear

Scheme 1. Overall Summary of Biotransformation of 3',4'-Anhydrovinblastine (3) to Bisindole Alkaloids.



that enzymatic control of the multi-step process from 1 and 2 and/or 3 to 9 and, in turn, to the alkaloids 4-8 must be realized through specific enzyme isolation before appreciable yields of end products can be obtained. We have already shown[1] that a high yielding enzymatic system for the coupling of 1 and 2 to afford 9 and finally 3 and 4 can be achieved through immobilization methods. It is hoped that further studies will achieve a similar system for vinblastine production.

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REFERENCES

1. Part XV. J. P. Kutney, C. A. Boulet, L. S. L. Choi, W. Gustowski, M. McHugh, J. Nakano, T. Nikaido, H. Tsukamoto, G. M. Hewitt, and R. Suen. 1987. Accompanying publication.
2. Part XIV. J. P. Kutney, C. A. Boulet, L. S. L. Choi, W. Gustowski, M. McHugh, J. Nakano, T. Nikaido, H. Tsukamoto, G. M. Hewitt, and R. Suen. 1987. Accompanying publication.
3. K. L. Stuart, J. P. Kutney, T. Honda, and B. R. Worth, Heterocycles, 1978, 9, 1419.
4. J. P. Kutney, L. S. L. Choi, T. Honda, N. G. Lewis, T. Sato, K. L. Stuart, and B. R. Worth, Helv. Chim. Acta, 1982, 65, 2088.
5. R. L. Baxter, C. A. Dorschel, S. L. Lee, and A. I Scott, J.C.S., Chem. Comm., 1979, 257.
6. R. L. Baxter, M. Hasan, N. E. MacKenzie, and A. I. Scott, J.C.S., Chem. Comm., 1982, 791.
7. J. P. Kutney, B. Aweryn, L. S. L. Choi, P. Kolodziejczyk, W. G. G. Kurz, K. B. Chatson, and F. Constabel, Heterocycles, 1981, 16, 1169.
8. J. P. Kutney, B. Aweryn, L. S. L. Choi, P. Kolodziejczyk, W. G. W. Kurz, K. B. Chatson, and F. Constabel, Helv. Chim. Acta, 1982, 65, 1271.
9. W. R. McLauchlan, M. Hasan, R. L. Baxter, and A. I. Scott, Tetrahedron, 1983, 39, 3777.
10. D. Neumann, G. Kraus, M. Hieke, and D. Groger, Planta Med., 1983, 48, 20.

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