ALTERED ALKALOID PATTERN IN DARK GROWN SEEDLINGS OF CATHARANTHUS ROSEUS. THE ISOLATION AND CHARACTERIZATION OF 4-DESACETOXYVINDOLINE[+]: A NOVEL INDOLE ALKALOID AND PROPOSED PRECURSOR OF VINDOLINE[‡]

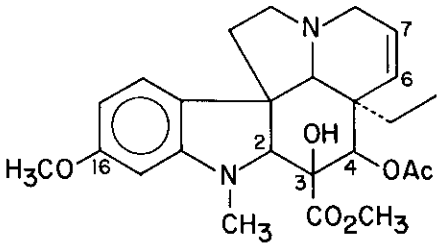
John Balsevich*, Vincenzo DeLuca, and Wolf G.W. Kurz
National Research Council of Canada
Plant Biotechnology Institute
Saskatoon, Saskatchewan
Canada, S7N 0W9

Abstract - Seeds of Catharanthus roseus cv. Little Delicata were germinated in the dark which resulted in relatively high and even rates of germination. Analysis of alkaloids from the cotyledons of 6 - 9 day old dark grown seedlings led to the identification of tabersonine, 16-methoxytabersonine, and catharanthine as relatively major components while vindoline, deacetylvindoline, 4-desacetoxyvindoline and 16-hydroxytabersonine were identified as minor components. Subjecting dark grown seedlings to light led to a rapid increase in the amount of vindoline present with a concomitant decrease in the amounts of tabersonine, 16-methoxytabersonine and the minor components. The amount of catharanthine present was not greatly affected. These observations suggested that the biosynthetic pathway between tabersonine and vindoline proceeds as follows: 16-hydroxytabersonine → 16-methoxytabersonine → 16-methoxy-2,3-dihydro-3-hydroxytabersonine → 16-methoxy-2,3-dihydro-3-hydroxy-N(1)-methyltabersonine (i.e. desacetoxyvindoline) → deacetylvindoline → vindoline.

[+] The numbering system used was as for aspidospermidine in Chemical Abstracts.

[‡] NRCC No. 25861

Lately the detailed pathway of vindoline (1) biosynthesis has come under scrutiny, largely as a consequence of the inability of cultured plant cells to produce it or the clinically important "dimeric" alkaloids to which it is a precursor.



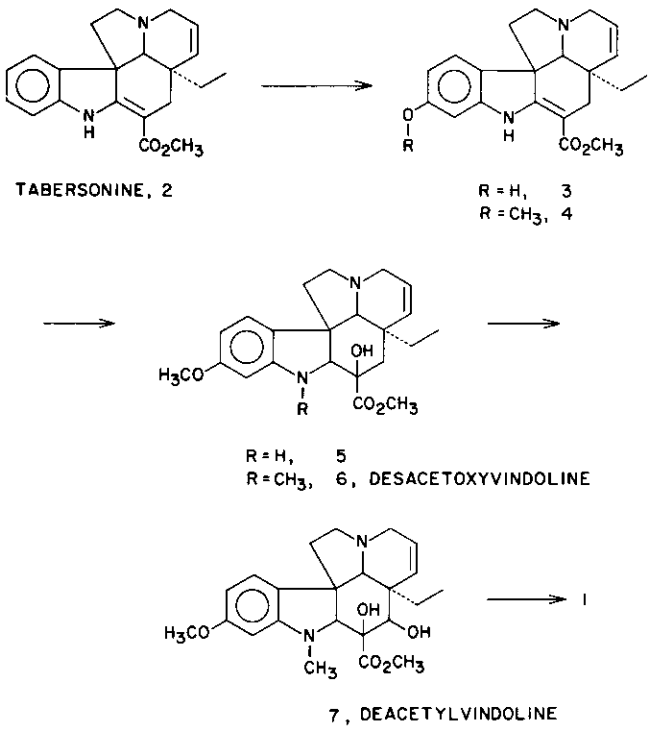
VINDOLINE, I

Although the biogenetic origin of vindoline from tabersonine (2) has long been established[1], the exact sequence of intermediate steps had previously not been determined. Recently, the last step in this pathway was shown by DeLuca et al.[2] to be the acetylation of deacetylvindoline by a specific acetyl transferase which exhibited end-product inhibition with respect to coenzyme A, but not to vindoline. This work was further corroborated by Fahn et al.[3] who also observed methyltransferase activities in cell-free preparations of C. roseus leaves. Based on substrate specificity studies, the latter authors postulated a pathway to vindoline initially involving three successive oxygenations of tabersonine (yielding 16-O-desmethyl-N-desmethyldeacetyl-vindoline) followed by N-methylation, O-methylation, and acetylation [4]. Recently however, results in our laboratory have suggested that this proposal is incorrect. Working with dark grown seedlings we have isolated several alkaloids which we believe are intermediates between tabersonine and vindoline. Included among these was the novel derivative desacetoxyvindoline** (6). Accordingly, we would like to present here our results on the characterization of this new alkaloid as well as on the identification of several other seedling alkaloids. Furthermore, based on some time-course and radiolabelling studies, we wish to propose an alternative pathway from tabersonine to vindoline which is in agreement with all observations obtained to date (Fig. 1).

When C. roseus seeds were germinated in the light, poor germination and uneven growth resulted, whereas in the dark a very high (> 90%) and reproducible germination rate, accompanied by good and even growth was achieved [7]. The alkaloid profile of the dark grown seedlings was found to differ from those of light or dark/light grown seedlings. To obtain information on the

---

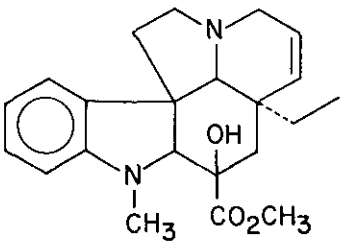
** Previously, Neuss et al. had reported the isolation of desacetoxyvinblastine from C. roseus plants[5]. Kutney et al.[6] have previously prepared 3-epidesacetoxyvindoline.



Scheme 1. Proposed biosynthetic pathway from tabersonine to vindoline.

alkaloids present 1 kg (fresh weight) of seedlings (maintained in the dark) were harvested 9 days after the seeds had been plated on agar (approx. 6 days after germination). Alkaloids were isolated (mainly from cotyledons) according to Kurz and Constabel[8] by which means 500 mg of an alkaloid mixture was obtained. The mixture was chromatographed on silica gel plates using ethyl acetate/methanol (8:1) as eluent yielding four "bands" of products (A-D in order of increasing polarity). Re-chromatography of each band on silica gel plates with the appropriate solvent led to the isolation of individual alkaloids having purities in excess of 85% (based on their ^{1}Hmr spectra). Thus, band A with petroleum ether/ether (2:1) gave tabersonine (2) and methoxytabersonine (4). Band B with ethyl acetate/methanol (20:1) yielded desacetoxyvindoline (6) and vindoline (1). Band C with ether/methanol (20:1) yielded catharanthine, while D with ethyl acetate/methanol (4:1) gave deacetylvindoline (7). With the exception of desacetoxyvindoline and 16-hydroxytabersonine (3), alkaloids were identified by comparison of their proton magnetic resonance and mass spectra as well as their chromatographic properties with those of authentic samples. 16-Hydroxytabersonine (3), which was present in trace amounts, was identified by its conversion to radiolabelled methoxytabersonine upon treatment with ^{14}C-labelled S-adenosyl

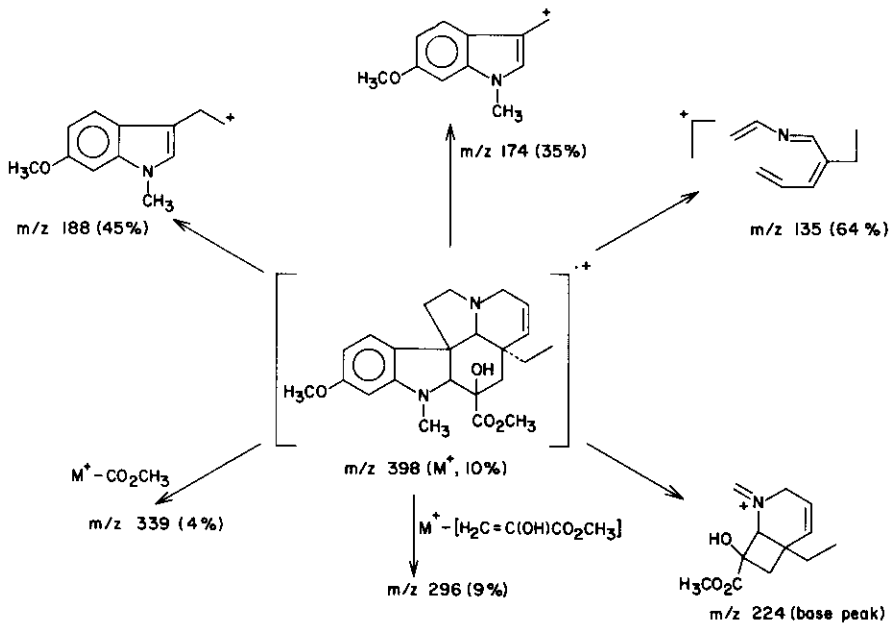
methionine (SAM) and an enzyme preparation, obtained from the mature plant[7]. Desacetoxyvindoline (6), a novel compound, was identified mainly on the basis of a comparison of its proton magnetic resonance and mass spectra with those of vindoline and desacetoxyvindorosine (8) (i.e. 16-desmethoxydesacetoxyvindoline)[9]. Thus, the mass spectrum of 6 exhibited a parent ion at



8

m/z 398 and several distinct fragments (Scheme 2), a number of which (m/z 196, 188, 174, 135) were common to vindoline and O-deacetylvindoline[10]. Furthermore, the peak at m/z 224, attributable to a fragment which contained C-3 and C-4, was also present in the spectrum of 4-desacetoxyvindorosine (8); the analogous fragments arising from vindoline and deacetylvindoline occurred at m/z 282 and 240 respectively. Conclusive verification of the proposed structure was obtained from the proton magnetic resonance spectrum which exhibited the following resonances: (360 MHz, $CDCl_3$) δ: 6.86 (1H,d, J=8.2 Hz), 6.25 (1H,dd, J=8.2 and 2.2 Hz), 6.02 (1H,d, J=2.2 Hz), 5.67 (1H,dd, J = 10 and 5.3 Hz), 5.45 (1H,brd, J=10 Hz), 3.78 (3H,s), 3.76 (3H,s), 3.63 (1H,d, J=1.7 Hz), 3.35 (1H,m), 2.76 (1H,bd, J=15.7Hz), 2.67 (3H,s), 2.52 (1H,s), 2.49 (2H,m), 2.27 (2H,m), 1.98 (1H,d of AB quartet, J=14.7 Hz), 1.91 (1H,dd of AB quartet, J=14.7 and 1.7 Hz), 1.13-0.88 (2H,m), 0.59 (3H,t, J=7.4 Hz). Aside from an extra three proton singlet at δ 3.76 and differences in the "aromatic region" the spectrum was virtually identical to that of desacetoxyvindorosine. In particular, the presence of the AB quartet centered about δ 1.95, attributable to the C-4 methylene group, established the presence of the tertiary hydroxy group at C-3 and ruled out the possibility of a secondary hydroxy group at C-4.

Having identified these aspidosperma alkaloids, their role as intermediates on the pathway to vindoline could be inferred from a qualitative comparison of alkaloid profiles (tlc) over a period of time, prior to and after transfer of dark grown seedlings to the light, as well as by a quantitative comparison of partial alkaloid profiles between dark and light grown seedlings at



Scheme 2. Mass spectral fragmentation of desacetoxyvindoline.

a single similar point in time (Table 1). Qualitatively, the transfer of dark grown (6 day) seedlings to light led to a rapid decrease (within 3 days) in the amount of tabersonine, hydroxytabersonine, methoxytabersonine, desacetoxyvindoline, and deacetylvindoline accompanied by an increase in the amount of vindoline. These observations were supported by the quantitative data (Table 1) which illustrated dramatically the reduced production of vindoline and the build-up of the other aspidosperma alkaloids in the dark grown seedlings. By comparison, production of catharanthine, an iboga alkaloid, was little changed.

To garner further evidence, leaf homogenates of mature plants were treated with radiolabelled SAM under various conditions in the hope that vindoline precursors would be present and lead to the production of later stage radiolabelled intermediates. This appeared to be the case. In the presence of added acetyl CoA and NADPH a radiolabelled compound which co-migrated with vindoline was obtained (tlc). In the absence of acetyl CoA and NADPH no radiolabelled vindoline was observed, instead a radiolabelled compound which co-migrated with desacetoxyvindoline was obtained. Although these results were preliminary, coupled with the previous observations they provided a strong case for the intermediacy of desacetoxyvindoline and the pathway outlined in Scheme 1.

Table 1. Comparison of amounts of alkaloids obtained from dark and light grown seedlings of Catharanthus roseus.

Alkaloid	Amount of Alkaloid[1] (mg/kg of seedling) fr. wt.	
	Dark Grown Seedlings	Light Grown[2] Seedlings
Tabersonine (2)	40	13
16-Methoxytabersonine (4)	15	10
16-Hydroxytabersonine (3)	trace	-
Desacetoxyvindoline (6)	10	trace
Vindoline (1)	5	54
Deacetylvindoline (7)	4	trace
Catharanthine	31	37

[1] Alkaloids were isolated mainly from cotyledons.
[2] Light grown seedlings were germinated (4 days) in the dark and then transferred to a lighted environment (4 days). Dark grown seedlings were germinated in the dark (4 days) and maintained in the dark (4 days).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Mr. B. Panchuk and helpful discussions with Dr. F. Constabel and Dr. R. Tyler.

REFERENCES

1. A.I. Scott, Acc. Chem. Res., 3, 151 (1970).
2. V. DeLuca, J. Balsevich and W.G.W. Kurz, J. Plant Physiol., 121, 417 (1985).
3. W. Fahn, H. Gundlach, B. Deus-Neumann, and J. Stockigt, Plant Cell Reports, 4, 333 (1985).
4. W. Fahn, E. Laussermair, B. Deus-Neumann and J. Stockigt, ibid., 337 (1985).
5. N. Neuss, A.J. Barnes and L.L. Huckstep, Experientia, 31, 18 (1975).
6. J.P. Kutney, J. Balsevich, T. Honda, P.-H. Liao, H.P.M. Thiellier, and B.R. Worth, Can. J. Chem., 56, 2560 (1978).

7. V. DeLuca, J. Balsevich, R.J. Tyler, U. Eilert, B.D. Panchuk, and W.G.W. Kurz, J. Plant Phys, in press (1986).

8. W.G.W. Kurz and F. Constabel, "Production and isolation of secondary metabolites" in "Plant Tissue Culture Methods", 2nd ed., L.R. Wetter and F. Constabel, eds., Natl. Res. Council of Canada, Ottawa, NRCC #19876, pp. 123-131 (1982).

9. G. Hugel, G. Massiot, J. Levy, and J. LeMen, Tetrahedron, 37, 1369 (1981).

10. N. Neuss, D.S. Fukuda, G.E. Mallett, D.R. Brannon, and L.L. Huckstep, Helv. Chim. Acta, 56, 2418 (1973).

Received, 19th May, 1986