FLAVINE COENZYME MEDIATED PHOTOOXIDATION OF 3',4'-ANHYDROVINBLASTINE. FURTHER INFORMATION ON THE LATER STAGES OF BISINDOLE ALKALOID BIOSYNTHESIS.

James Peter Kutney*, Lewis Siu Leung Choi, Jun Nakano, and Hiroki Tsukamoto

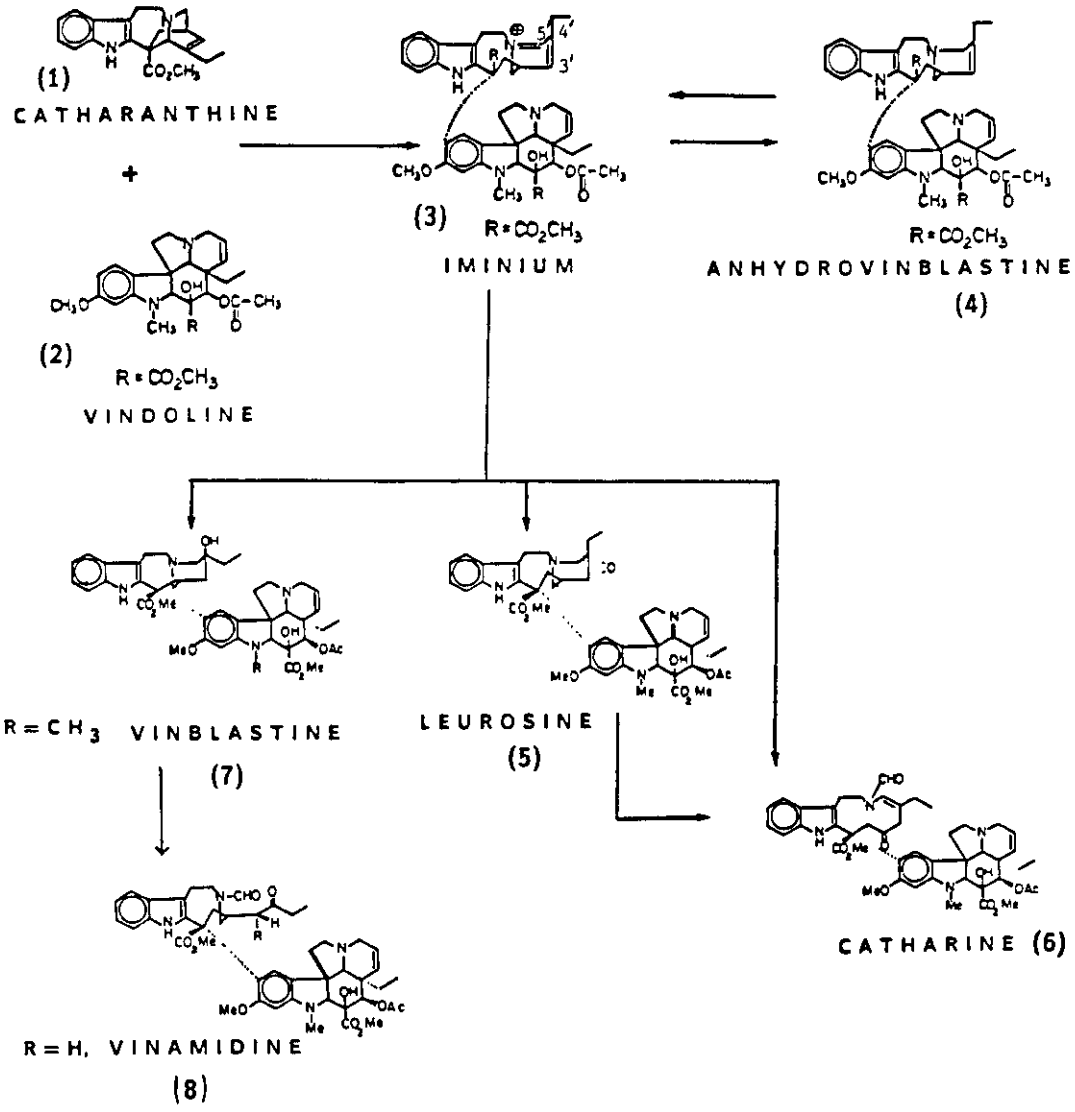
Department of Chemistry, The University of British Columbia, 2036 Main Mall, Vancouver, B. C., Canada V6T 1Y6

Abstract - Flavine coenzyme mediated photooxidation of 3',4'-anhydrovinblastine (4) has afforded an excellent route to the highly unstable dihydropyridinium intermediate 3. The role of 3 as the initially formed biointermediate in the enzyme-catalyzed coupling of catharanthine (1) and vindoline (2) can now be evaluated in terms of its crucial role in the biosynthesis of the various bisindole alkaloids such as 4, leurosine (5), catharine (6), vinblastine (7), and vinamidine (8). Extensive studies are presented to establish the structure and chemistry of 3. Techniques such as ^{13}C-nmr and Attached Proton Test (APT) are employed. It is also shown that in the non-enzymatic FMN conversion of 4 to 3, the process proceeds with preferential removal of the C5'-αH in the indole unit of 4 (66% removal in deuterio-labelled as substrate) while in the comparable enzymatic conversion predominant retention of this proton is maintained (65% retention of C5'-αH in deuterio-labelled 4). A similar study in the bioconversion of 4 to 3 with commercial horseradish peroxidase reveals a 50% removal of this proton. Clearly the nature of the stereoselectivity of the enzymatic process in the generation of 3 is dependent on the nature of oxidases/peroxidases present in the enzyme mixture employed. These studies also provide mechanistic information about the one-electron promoted bioconversion of 4 to 3.

The role of 3',4'-anhydrovinblastine (4) as a possible intermediate in the biosynthesis of the bisindole alkaloids has been discussed in studies from various laboratories. In our earlier

studies with cell free enzyme systems from Catharanthus roseus plants[1-4], we could show that 4 is obtained from the coupling of catharanthine (1) and vindoline (2) and that 4, in turn, is converted to the bisindole alkaloids leurosine (5), catharine (6) and vinblastine (7). An independent study by Scott and co-workers[5,6] provided a similar result.

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



In speculating upon a possible mechanistic pathway to 4, we proposed in 1978[3] that an unstable dihydropyridinium intermediate 3 could be involved. Subsequent studies employing C. roseus cell cultures[7-9] evaluated the biotransformation of 4 to other metabolites and these results cast further light on the role of 3 in this area of biosynthesis. Our most recent and very extensive studies[10-12] with C. roseus whole cells, "spent" medium, crude cell free enzyme systems and immobilized enzyme systems derived from various ages of C. roseus cell cultures have provided still further insight into the intermediacy, if any, of 4 in the biosynthesis of the above-noted alkaloids. With respect to the present discussion, the most significant result of these latter studies was the unambiguous detection of the same unstable dihydropyridinium intermediate 3 formed in the biotransformation of 4 and the enzyme-catalyzed coupling of 1 and 2. Since the enzymes responsible for the biotransformation of 4 are activated by the use of FMN or FAD as coenzymes, it was considered important to evaluate the transformation of 4 with these coenzyme systems in order to shed light on the possible mechanism of this biotransformation.

To a thoroughly degassed solution of 4 (hydrogen sulfate salt) in methanol and Tris-HCl buffer (pH 7 2) under an argon atmosphere, was added a degassed solution of FMN in Tris-HCl buffer (pH 7.2). Irradiation with white fluorescent light at room temperature for 6 h revealed, via HPLC monitoring according to a previously established procedure[10], disappearance of 4 and appearance of the dihydropyridinium intermediate 3. It was mandatory to use oxygen-free conditions in order to avoid complex product formation from the highly reactive and unstable 3. After complete conversion of 4 to 3, rapid reductive workup ($NaBH_4$) and extraction under argon was required to obtain a good recovery of regenerated 4 (>80%) Although no reaction was observed in the absence of FMN (or FAD), 4 was highly unstable towards the FMN/$NaBH_4$ aqueous mixture present in the workup process. Over 90% of 4 was converted to a complex mixture of products only 2 minutes after addition of a Tris-HCl buffer solution of FMN/$NaBH_4$.

The assignment of the conjugated dihydropyridinium structure 3 for the FMN-mediated photooxidation product of 4 was ascertained by means of a deuterium incorporation experiment. After photooxidation of 4 was completed as described above, the reduction was carried out with $NaBD_4$ instead of $NaBH_4$ The product obtained was recrystallized from methanol, and calculation from mass spectroscopic measurements, indicated a high incorporation (92%) of one deuterium into the resultant deuterio-3',4'-anhydrovinblastine ([^{2}H]-4).

The position(s) of deuterium incorporation was determined by nmr (^{1}H and ^{13}C) spectral analyses. The ^{1}H-nmr spectrum of the deuterio product was identical in every respect with that of authentic 4 except for the disappearance of the normal doublet centered at δ 3.52 (J = 16 Hz), which has been assigned to C5'-αH. At the same time, the corresponding C5'-βH signal in the deuterated product appeared as a singlet at δ 3.25 instead of the usual doublet (δ 3 27, J = 16 Hz) seen in the spectrum of undeuterated 4. This assignment was further confirmed by a proton decoupling study when on irradiation of the C3' vinyl proton (δ 5.46, br. s), an increase in intensity of the new singlet (δ 3.25) was observed. These data are in agreement with the assigned structure 3 for the FMN mediated photooxidation product. The latter on treatment with $NaBD_4$ underwent a 1,2-reduction of the iminium double bond ($\rightleftharpoons \overset{\oplus}{N}=C5' \rightleftharpoons$) with high stereoselective incorporation of deuterium occurring mainly at the α-face of the 5'-carbon because of steric hindrance from the β-side. Similar high stereoselectivity in the $NaBD_4$ reduction of 3 generated by the modified Polonovski coupling of (1) and (2), was reported by Scott et al[6].

The lack of deuterium incorporation at all the other positions of the regenerated deuterio 3',4'-anhydrovinblastine except for the 5' carbon was confirmed by ^{13}C-nmr spectroscopic analysis. As shown in Tables I and II, the deuterio 3',4'-anhydrovinblastine ([^{2}H]-4) regenerated from the photooxidation product and authentic 4 have identical ^{13}C-nmr spectra except for the signals corresponding to the 5' carbon in the indole unit. Thus in 4, a resonance appeared as a singlet at 52.1 ppm while for [^{2}H]-4 the C5' signal appeared as a group of multiplets (most likely a set of triplets) at 51.75 ppm to 51.45 ppm. The Attached Proton Test (APT)[13-15], that was obtained in conjunction with the normal ^{13}C-nmr spectrum, was also of diagnostic value. In the ^{13}C-APT spectrum, the C5' singlet for 4 appeared as a positive (upward) signal corresponding to a methylene carbon with 2 attached protons while the ^{13}C-APT signal for C5' of [^{2}H]-4 appeared as a group of negative (downward) multiplets (51.75 ppm to 51.45 ppm) in accord with a methylene carbon attached to 1 proton and 1 deuterium.

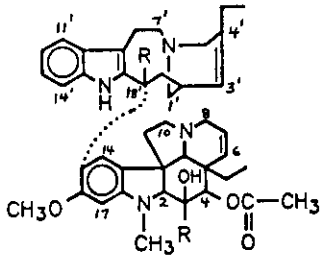
The result of the above deuterium incorporation study clearly established the structure of the flavine mediated photooxidation product as the conjugated iminium ion 3. No deuterium incorporation was detected at C19' and C7'. Similarly, for the vindoline moiety of ([^{2}H]-4), there was no evidence of deuterium incorporation in any of the carbon atoms adjacent to the two tertiary nitrogen atoms, thereby excluding FMN-mediated photooxidation in the vindoline unit.

Table 1

^{13}C-Nmr chemical shifts and Attached Proton Test (APT) for the indole unit of 3',4'-anhydrovinblastine (4) and deuterio-3',4'-anhydrovinblastine.

		3',4'-Anhydrovinblastine		Deuterio-3',4'-Anhydrovinblastine	
Carbon Number	Number of Proton Attached	Chemical Shift (ppm)[a]	APT[b]	Chemical Shift (ppm)[a]	APT[b]
1'	2	34.3	+	34.2	+
2'	1	32.9	-	32.8	-
3'	1	123.7	-	123.6	-
4'	0	139.8	+	139.6	+
5'	(2	52.1	+		
	(1 (+1D)			51.45-51.75 (multiplet)	-
7'	2	54.4	+	54.4	+
8'	2	25.5	+	25.5	+
9'	0	117.3	+	117.2	+
10'	0	129.3	+	129.2	+
11'	1	118.2	-	118.1	-
12'	1	122.1	-	122.0	-
13'	1	118.7	-	118.6	-
14'	1	110.4	-	110.3	-
15'	0	134.8	+	134.7	+
17'	0	130.7	+	130.7	+
18'	0	55.4	+	55.3	+
19'	2	45.7	+	45.7	+
20'	3	12.3	-	12.2	-
21'	2	27.7	+	27.7	+
C=O	0	174.5	+	174.4	+
O-Me	3	52.4	-	52.3	-

[a] Downward from TMS
[b] + indicated upward signal; - indicated downward signal.



R=CO_2CH_3

4

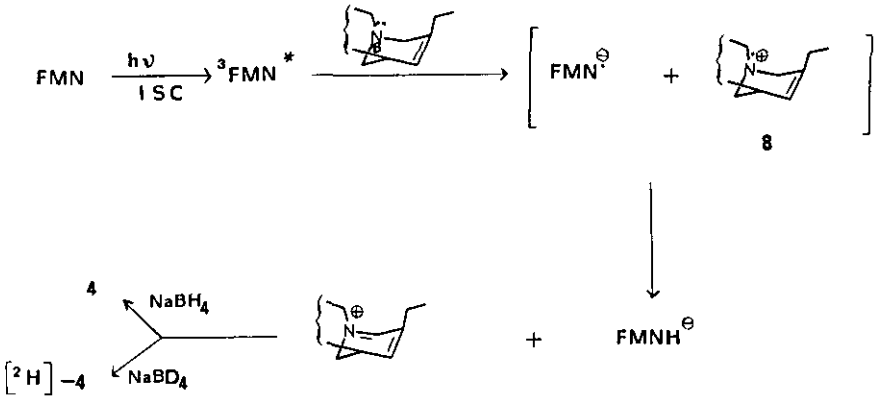
Table 2

^{13}C-Nmr chemical shifts and Attached Proton Test (APT) for the vindoline half of 3',4'-anhydrovinblastine (4) and deuterio-3',4'-anhydrovinblastine.

		3',4'-Anhydrovinblastine		Deuterio-3',4'-Anhydrovinblastine	
Carbon Number	Number of Proton Attached	Chemical Shift (ppm)[a]	APT[b]	Chemical Shift (ppm)[a]	APT[b]
2	1	83.2	-	83.2	-
3	0	79.6	+	79.5	+
4	1	76.4	-	76.3	-
5	0	42.6	+	42.5	+
6	1	129.8	-	129.7	-
7	1	124.4	-	124.3	-
8	2	50.2	+	50.1	+
10	2	50.2	+	50.1	+
11	2	44.6	+	44 5	+
12	0	53.2	+	53.2	+
13	0	122.6	+	122.5	+
14	1	123.4	-	123.3	-
15	0	121.1	+	121.0	+
16	0	157.8	+	157.7	+
17	1	94.1	-	94.0	-
18	0	152.5	+	152.4	+
19	1	65.3	-	65.2	-
20	3	8.4	-	8.3	-
21	2	30.8	+	30.7	+
C3-C=O	0	170.7	+	170.6	+
C3-CO_2Me	3	52.2	-	52.1	-
C4-O-C=O	0	171.5	+	171.4	+
C4-OCOMe	3	21.2	-	21.1	-
C16-OMe	3	55.8	-	55.8	-
N_1-Me	3	38.4	-	38.3	-

[a] Downfield from TMS
[b] + indicated upward signal; - indicated downward signal.

Mechanistically, the requirement of both FMN (or FAD) and white fluorescent light irradiation for the anaerobic oxidation of 4 to proceed, strongly suggests that the initial step of the reaction involves the excitation of the flavin molecule as outlined in Scheme 2. The triplet state flavin ($^3FMN^*$) formed by inter-system crossing (ISC) is then quenched by abstracting an electron from the nitrogen atom at the 6-position to afford a radical-ion pair (8). Transfer of a C5' hydrogen within the radical-ion pair complex then produces the conjugated iminium ion 3. Sodium borohydride reduction of the latter would regenerate 4 while reduction with $NaBD_4$ results in [^{2}H]-4 with high stereoselective deuterium incorporation occurring at the less hindered α face of C5'.



Scheme 2

The initial steps of the above proposed mechanism are analogous with that established for the flavin-mediated photodecarboxylation of certain heteroatom-substituted carboxylic acids[16].

The results of our recent biotransformation of 4, utilizing enzyme systems from our C. roseus cell cultures[12], have established the conjugated dihydropyridinium ion 3 as the crucial intermediate from 4 to the various bisindole alkaloids. In these latter studies, flavin co-enzymes were also found to be important co-factors for the biotransformation of 4 so the present study may bear a close relationship to the enzymatic biotransformation of 4 Consequently, it was of interest to examine the stereochemistry of hydrogen elimination from C5' of 4 in the non-enzymatic flavin mediated photooxidation and to compare the data with those involving the biotransformation of 4 by enzyme systems derived from C. roseus cell

cultures. Employing deuterio 3',4'-anhydrovinblastine labelled at C5'-α as the starting compound[17], a FMN mediated photooxidation was performed as described above to form the conjugated dihydropyridinium intermediate 3. The latter was reduced with $NaBH_4$ and the regenerated 3',4'-anhydrovinblastine (4), after purification, was found by mass spectroscopic measurements to retain 34% of the original deuterium label. This result indicated that under the conditions employed, loss of the C5'α-hydrogen is favored over the alternative C5'β-H in the FMN mediated photooxidation by a ratio of 2:1. The incubation of C5'-α-deuterio-3',4'-anhydrovinblastine with cell free extracts from an 8 day old C. roseus cell cultures[18] was carried out in the absence of any added flavin co-factors in order to eliminate any possible non-enzymatic transformation. Conversion to the dihydropyridinium ion 3 was monitored by HPLC and after 96 h, the accumulated 3 was reduced with $NaBH_4$. The regenerated 3',4'-anhydrovinblastine (4) was found by mass spectroscopic measurements to retain 65% of the original deuterium label. To further eliminate any non-enzymatic photooxidation of 4 mediated by endogenous flavin co-factors present in the cell free extracts, an incubation of C5'-α-deuterio 4 was carried out in the dark. Similar conversion to 3, as monitored by HPLC, was observed and after $NaBH_4$ reduction, the regenerated 4 was found to retain 67% of the original deuterium label. These results indicated that in the cell free enzymatic transformation of 4 to 3 elimination of the C5'-β hydrogen is favored over the C5'-α hydrogen by a ratio of 2:1, directly opposite to that observed in the FMN mediated reaction.

The apparent lack of complete stereospecificity in the enzymatic formation of 3 from 4 by cell free extracts derived from C. roseus cell cultures can be rationalized by the fact that the crude cell free extracts employed contained many enzymes (peroxidases and/or oxidases)[10-12]. Some of the enzymes may be specific but others may not, so that the above biotransformation of 4 to 3 represented the overall effects by the total enzyme mixture. In this regard, it is of interest to note that 3',4'-anhydrovinblastine (4) can also undergo a facile conversion to 3 by the enzymic action of commercially available horseradish peroxidase (HRP)[19] in the presence of a controlled amount of H_2O_2. Earlier we had reported the facile enzymic conversion of 4 to leurosine (5) by both HRP and cell free extracts derived from C. roseus leaves in the presense of excess H_2O_2[8]. By means of HPLC monitoring, it is now possible to control the HRP/H_2O_2 transformation of 4 to a mixture of 3 and 5 in 60 min in Tris-HCl buffer (pH 6.8). It was then of interest to determine the stereochemistry of the hydrogen elimination from C5' of 4 in this HRP catalyzed reaction. Employing the same C5'-α-deuterio anhydrovinblastine as the substrate, a mixture of 3 and 5 was obtained on treatment with HRP/H_2O_2. Reduction of the reaction mixture with $NaBH_4$ then regenerated 3',4'-anhydrovinblastine which, after purification, was

found to retain 50% of the original deuterium label. This result demonstrated that commercially available peroxidase (HRP) can readily transfer 4 to 3 but also not with high stereospecificity.

Recently, Rosazza et al.[20] reported a one-electron oxidation of vindoline (2) and its derivatives catalyzed by HRP. A highly reactive radical cation species of 2 was an implied intermediate in the oxidation process. We believe that a one-electron oxidation process is also operative in both our cell free enzymic extracts and the HRP-catalyzed transformation of 4. A radical cation similar to 8 is the first formed intermediate which can undergo a stereospecific transfer of a C5' hydrogen within an enzyme-substrate complex, or it can eliminate a second electron and a proton non-stereospecifically to form 3.

The presence of specific enzyme systems in the cell free extracts of C. roseus was implicated also in the results reported by Scott et al.[6] in the biotransformation of 4 to vinblastine (7) by cell-free extracts from C. roseus leaves. Although the yield to 7 was low (1.67%), they observed a high retention of the C5'-αH labelled as tritium in the product (7) obtained.

In summary, the present study clearly establishes that the enzyme mediated biotransformation of 3',4'-anhydrovinblastine (4) to the crucial biosynthetic intermediate 3 follows a pathway similar to that of the FMN-mediated conversion of 4 to 3 in a non-enzymatic experiment. The much higher specificity of C5'-H removal in the enzymatic conversion is of interest. Furthermore, the conversion of 4 to 3 can occur in the absence of FMN although the process, at least with enzymes obtained from an 8 day old culture, is much slower. Thus the cofactor dependency of FMN in the enzymatic conversion is established. Finally, it is clear that 4 is a product obtained after enzymatic reduction of 3, the latter being formed in the coupling of 1 and 2. However, if 4 is incubated as the substrate in an enzymatic process, it is converted back to 3 and the latter then converts to the various bisindole alkaloids. The results obtained in this study are applied to other experiments as described in the accompanying publications [21,22]

ACKNOWLEDGEMENTS

Financial aid from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

REFERENCES

1. K. L. Stuart, J. P. Kutney, and B. R. Worth, Heterocycles, 1978, 9, 1015.

2. K. L. Stuart, J. P. Kutney, T. Honda, and B. R. Worth, Heterocycles, 1978, 9, 1391.

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. W. 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. 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, Heterocycles, 1988, 27, 613.

11 J P Kutney, C A Boulet, L. S. L. Choi, W. Gustowski, M. McHugh, J. Nakano, T. Nikaido, H. Tsukamato, G. M. Hewitt, and R. Suen, Heterocycles, 1988, 27, 621.

12. J. P. Kutney, B Botta, C. A. Boulet, C. A. Buschi, L. S. L. Choi, J. Golinski, M. Gumulka, G M. Hewitt, G. C. Lee, M. McHugh, J. Nakano, T. Nikaido, J. Onodera, I. Perez, P. J. Salisbury, M. Singh, R. Suen, and H. Tsukamoto, Heterocycles, 1988, 27, 629.

13 S. L. Patt, and J. N. Shoolery, J. Magnetic Resonance, 1982, 46, 535.

14 C Lecocq, and L. Y Lallemand, J. C. S., Chem. Comm., 1981, 150.

15 D. L. Rabenstein, and T. T. Nakashima, Anal. Chem., 1979, 51, 1465.

16 T C. Bruice, Acc. Chem. Res., 1980, 13, 256.

17. [5'α- H] Anhydrovinblastine (95% deuterium incorporation) was prepared by the modified Polonovski coupling of 1 and 2 and reduction of the resultant conjugated iminium intermediate with $NaBD_4$.

18. Crude cell free extracts were prepared as described in ref. 10.

19. Type VI HRP (Sigma Chemical Co., St. Louis, MO, U.S.A.).

20 F Sima Sariaslani, M. W Duffel and J. P. Rosazza, J. Med. Chem., 1985, 28, 629.

21. J. P. Kutney, L. S. L. Choi, J. Nakano, and H. Tsukamoto, Heterocycles, 1988, accompanying publication.

22. J. P. Kutney, L. S. L Choi, J. Nakano, H. Tsukamoto, M. McHugh, and C. A. Boulet, Heterocycles, 1988, accompanying publication.

Received, 18th April, 1988