tamic acid, 1.1; proline, 1.0; glycine, 1.0; cystine, 0.27; mixed disulfide of cysteine and N-methylcysteine, 0.34; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.9; and ammonia, 3.1. The values for cystine and the mixed disulfide account for the half-cystine residue in this analog.[22]

"Acetone-N-methyl-oxytocin." A sample (138 mg) of N-methyl-oxytocin that had not been treated with acetone was dissolved in 80% aqueous acetone (138 ml) and allowed to stand at room temperature in the dark for 7 days. The product isolated by evaporation and lyophilization was dissolved in 5 ml of the organic phase of solvent system A and subjected to partition chromatography in solvent system A on a Sephadex column (2.83 × 53.9 cm). The resulting chromatogram showed two peaks with R_f values of 0.76 and 0.25, the latter corresponding to N-methyl-oxytocin. Isolation of the materials represented by the peaks gave 84.3 mg of the fast-moving substance (R_f 0.76) and 37.2 mg of N-methyl-oxytocin. The material from the fast-moving peak was dissolved in 8 ml of the organic phase of solvent system B and subjected to partition chromatography in solvent system B on a Sephadex column (2.83 × 53.8 cm). The resulting chromatogram showed two peaks with R_f values of 0.82 and 0.53 with the latter being the major peak. The material represented by the major peak (61.4 mg) was dissolved in 4 ml of the organic phase of solvent system B and rechromatographed in solvent system B. The resulting chromatogram showed one peak with R_f 0.53 with a slight shoulder on the leading edge. Isolation of material corresponding to the central portion of the peak gave 42.7 mg. For further purification a sample (31.3 mg) of this material was dissolved in 0.2 *N* acetic acid (5 ml) and subjected to gel filtration on a Sephadex G-25 column (2.82 × 62.2 cm) in 0.2 *N* acetic acid. Isolation of material corresponding to the central portion of the major peak (maximum at effluent volume 343 ml) in the resulting chromatogram gave 24.1 mg of highly purified acetone-N-methyl-oxytocin, $[\alpha]^{21}D$ −136° (*c* 0.5, 1 *N* acetic acid). For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* with a loss in weight of 4.0%.

Anal. Calcd for $C_{47}H_{72}N_{12}O_{12}S_2$: C, 53.2; H, 6.84; N, 15.8. Found: C, 53.1, H, 6.93; N, 15.6.

A sample was hydrolyzed in 6 *N* HCl *in vacuo* at 110° for 48 hr and analyzed in a Beckman-Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were found, with the value of glycine taken as unity: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; cystine, 0.28; mixed disulfide of cysteine and N-methylcysteine, 0.36; leucine, 1.0; isoleucine, 1.1; tyrosine, 0.9; and ammonia, 3.0. These values are the same, within the experimental error, as those found for N-methyl-oxytocin.[22]

As in previous studies with "acetone-oxytocin,"[11] the liberation of acetone from the isopropylidene derivative of N-methyl-oxytocin was demonstrated by heating a sample with 2,4-dinitrophenylhydrazine in dilute methanolic HCl at 90° for 20 min and identification by paper chromatography of the acetone 2,4-dinitrophenylhydrazone so obtained. The quantitative determination of acetone liberated by the derivative was also carried out according to the procedure described previously.[11] When a sample (1.017 mg) was boiled in 0.1 *N* acetic acid (5 ml) for 6 min, the amount of acetone liberated was 25% of that theoretically obtainable from a monoisopropylidene derivative of N-methyl-oxytocin. When a sample (0.857 mg) was heated in 0.1 *N* acetic acid (1.2 ml) in a sealed tube at 95° for 20 hr, the amount of acetone liberated was 98% of the theoretical value.

On paper electrophoresis in pyridine acetate buffer of pH 5.6 (18 hr at 4° and 300 V) the isopropylidene derivative of N-methyl-oxytocin migrated toward the cathode at a considerably slower rate than N-methyl-oxytocin, as detected by color development with the Pauly reagent.

Acknowledgments. The authors are indebted to Mr. Joseph Albert for the elemental analyses, Mr. Roger Sebbane for the amino acid analyses, and Mrs. Jessie Lawrence and Miss Margita Wahrenburg for the bioassays, carried out under the direction of Dr. W. Y. Chan.

(22) The position and color value for the mixed disulfide of cysteine and N-methylcysteine was obtained by analysis of an equimolar mixture of L-cystine and N,N'-dimethyl-L-cystine (see ref 20) after the mixture had been heated in 6 *N* HCl *in vacuo* at 110° for 48 hr. In the analysis of N-methyl-oxytocin the mixed disulfide appeared just ahead of the glutamic acid peak and a small shoulder containing N,N'-dimethyl-L-cystine appeared on the leading edge of the aspartic acid peak.

Biosynthesis of Indole Alkaloids. Vindoline[1]

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Abstract: The biosynthesis of vindoline in *Vinca rosea* plants has been investigated using appropriate radioactive precursors. The results obtained are in full accord with the suggested monoterpenoid origin for the nontryptophan-derived portion of the indole alkaloids.

In spite of the structural diversity shown by indole alkaloids[2,3] it has become increasingly obvious that most of them can be conveniently classified into three main structural groups.[4] These are described as the corynanthe, aspidosperma, and iboga groups and may be structurally derived (Figure 1) by combination of a tryptamine residue with C_{10} units (**1**, **2**, and **3**, respectively). Ajmaline (**4**), vindoline (**5**), and catharanthine (**6**) are shown as representative examples of each of the main groups. Structural variations within each group are numerous, but in spite of this the basic patterns are discernible. Certain alkaloids contain a C_9 unit in addition to the tryptamine residue, and it is invariably the carbon atom indicated by the dotted lines in **1**, **2**, and **3** which is lost.

The reality of this structural classification (Figure 1) in biosynthetic terms has been extensively probed, and

(1) Much of this work has been described briefly in communications: (a) T. Money, I. G. Wright, F. McCapra, and A. I. Scott, *Proc. Natl. Acad. Sci. U. S.*, **53**, 901 (1965); (b) F. McCapra, T. Money, A. I. Scott, and I. G. Wright, *Chem. Commun.*, 537 (1965); (c) E. S. Hall, F. McCapra, T. Money, K. Fukumoto, J. R. Hanson, B. S. Mootoo, G. T. Phillips, and A. I. Scott, *ibid.*, 348 (1966).

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(3) R. H. F. Manske, Ed., "The Alkaloids," Vol. VIII, Academic Press Inc., New York, N. Y., 1965.

(4) E. Schlittler and W. I. Taylor, *Experientia*, **16**, 244 (1960).

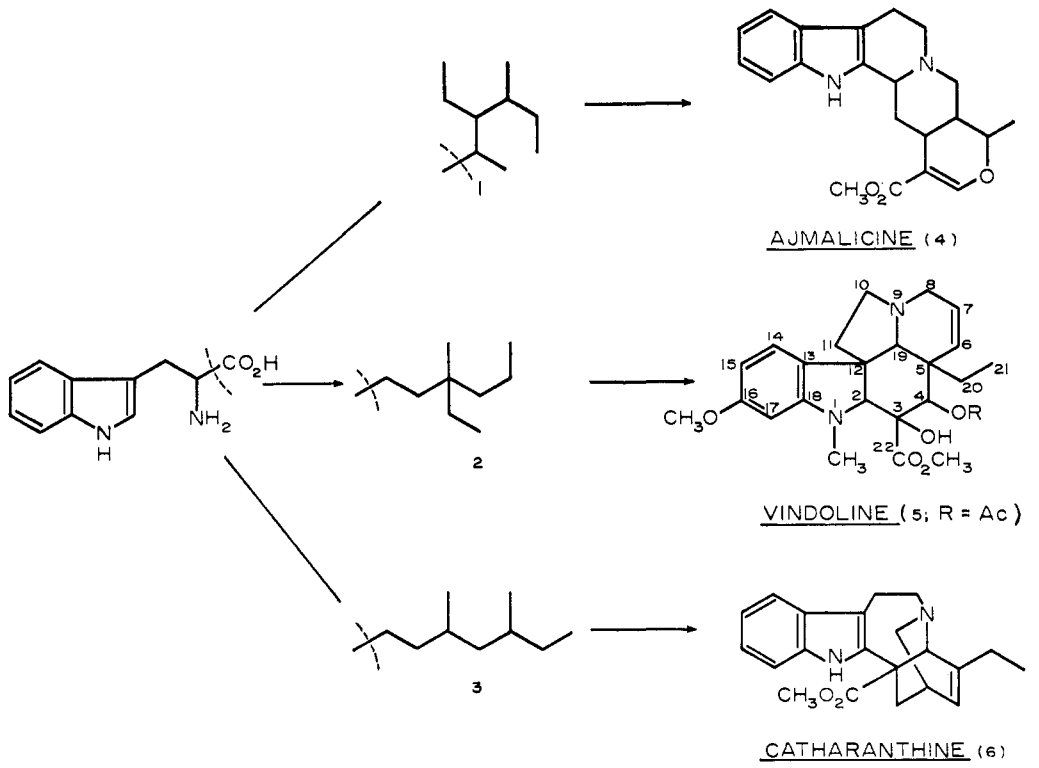

Figure 1.

indeed the expected derivation of the tryptamine unit from tryptophan has been demonstrated[5-9] for representative examples of each class of indole alkaloid. In contrast to these results, however, the origin of the C_9–C_{10} residue remained obscure. Several excellent biogenetic proposals[10-16] were made to account for the derivation of this portion of the indole alkaloid framework, and each of these was subjected to experimental study, using appropriate radiolabeled precursors. At the outset of our investigations (1964) the combined evidence from these experimental investigations had been presented[16-19a] and seemed to invalidate each of the biogenetic proposals which had been made. Later work[9,19b-22] published in 1965 supported this view and tended to suggest that a new hypothesis might be necessary to explain the accumulated radiochemical evidence.

A consideration of the evidence, however, revealed that the attractive proposals made independently by Thomas[15] and Wenkert[14c] (monoterpenoid hypothesis (Figure 2)) were rejected as a result of experiments in which mevalonic acid was *not* incorporated into specific indole alkaloids (*e.g.*, ajmaline).[17] The attractive rationale provided by the monoterpenoid hypothesis for the biosynthesis of the ubiquitous C_9–C_{10} portion of the indole alkaloids prompted us to reexamine this evidence. We were encouraged in this pursuit by observations made in the diterpene alkaloid field. Thus Kirby[23] had shown that mevalonic acid was not incorporated by *Delphinium elatum* plants into the diterpene alkaloid delpheline. This surprising result prompted him to recommend that in certain cases the negative incorporation of mevalonic acid by alkaloid-producing plants should be treated with caution. Vindication of this cautionary view was provided by Benn and May[24] who recorded the positive incorporation of mevalonic acid into the diterpene alkaloids lycoctonine and browniine. The latter workers also provided a reasonable explanation for the negative results recorded earlier.

We therefore elected to retest the monoterpenoid hypothesis and in particular the precursor activity of mevalonic acid. For this purpose we chose the indole alkaloid producing plant *Vinca rosea* (*Catharanthus roseus* G. Don)[25] and investigated the biosynthetic origin of the C_{10} unit of one of its major alkaloids,

(5) E. Leete, *Chem. Ind.* (London), 692 (1960); *J. Am. Chem. Soc.*, **82**, 6338 (1960).
(6) E. Leete, *Tetrahedron Letters*, 35 (1961).
(7) M. Yamasaki and E. Leete, *ibid.*, 1499 (1964).
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(9) E. Leete, A. Ahmed, and I. Kompis, *J. Am. Chem. Soc.*, **87**, 1468 (1965).
(10) G. Barger and C. Scholz, *Helv. Chim. Acta*, **16**, 1343 (1933).
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(13) R. Robinson, "Festschrift Arthur Stoll," Birhäuser, Basel, 1957, p 457; "The Structural Relations of Natural Products," Clarendon Press, Oxford, 1955, p 110.
(14) (a) E. Wenkert and N. V. Bringi, *J. Am. Chem. Soc.*, **81**, 1474 (1959); (b) E. Wenkert, *Experientia*, **15**, 165 (1959); (c) E. Wenkert, *J. Am. Chem. Soc.*, **84**, 98 (1962).
(15) R. Thomas, *Tetrahedron Letters*, 544 (1961).
(16) E. Leete and S. Ghosal, *ibid.*, 1179 (1962).
(17) E. Leete, S. Ghosal, and P. N. Edwards, *J. Am. Chem. Soc.*, **84**, 1068 (1962).
(18) P. N. Edwards and E. Leete, *Chem. Ind.* (London), 1666 (1961).
(19) (a) A. R. Battersby, R. Binks, W. Lawrie, G. V. Parry, and B. G. Webster, *Proc. Chem. Soc.*, 369 (1963); (b) *J. Chem. Soc.*, 7459 (1965).
(20) K. Stolle, D. Gröger, and K. Mothes, *Chem. Ind.* (London), 2065 (1965).
(21) D. H. R. Barton, G. W. Kirby, R. H. Prager, and E. M. Wilson, *J. Chem. Soc.*, 3990 (1965).
(22) H. Goeggel and D. Arigoni, *Experientia*, **21**, 369 (1965).
(23) E. J. Herbert and G. W. Kirby, *Tetrahedron Letters*, 1505 (1963).
(24) M. H. Benn and J. E. May, *Experientia*, **20**, 252 (1964).
(25) *Cf.* G. H. Svoboda, I. S. Johnson, M. Gorman, and N. Neuss, *J. Pharm. Sci.*, **51**, 707 (1962); N. Neuss, *Bull. Soc. Chim. France*, 1509 (1963).

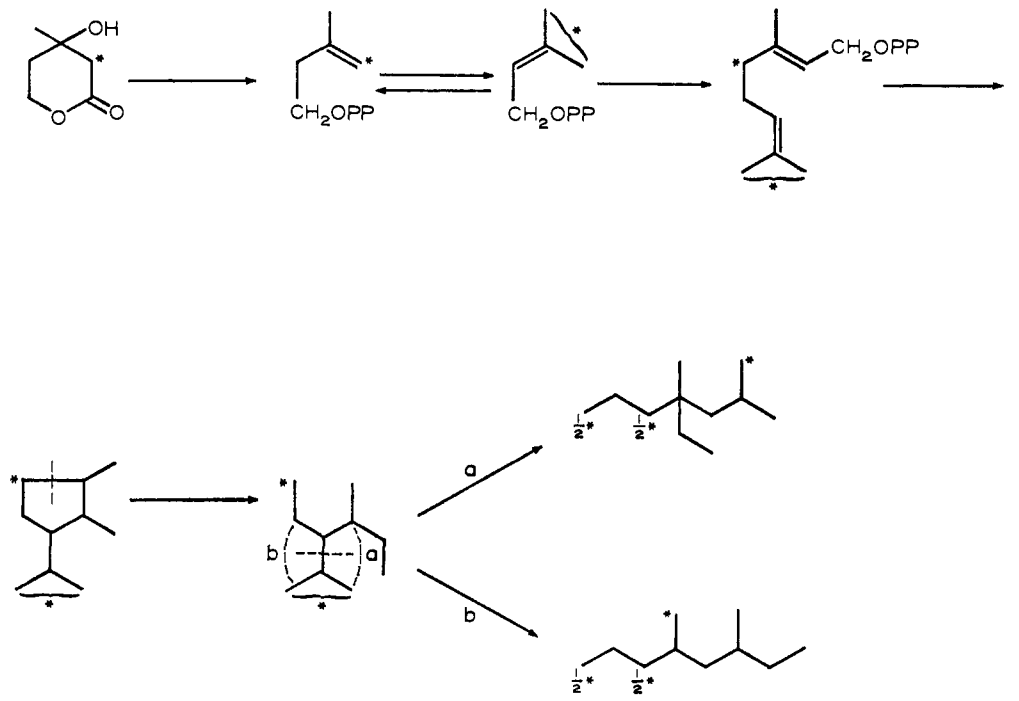


Figure 2. Thomas-Wenkert monoterpenoid theory (schematic).

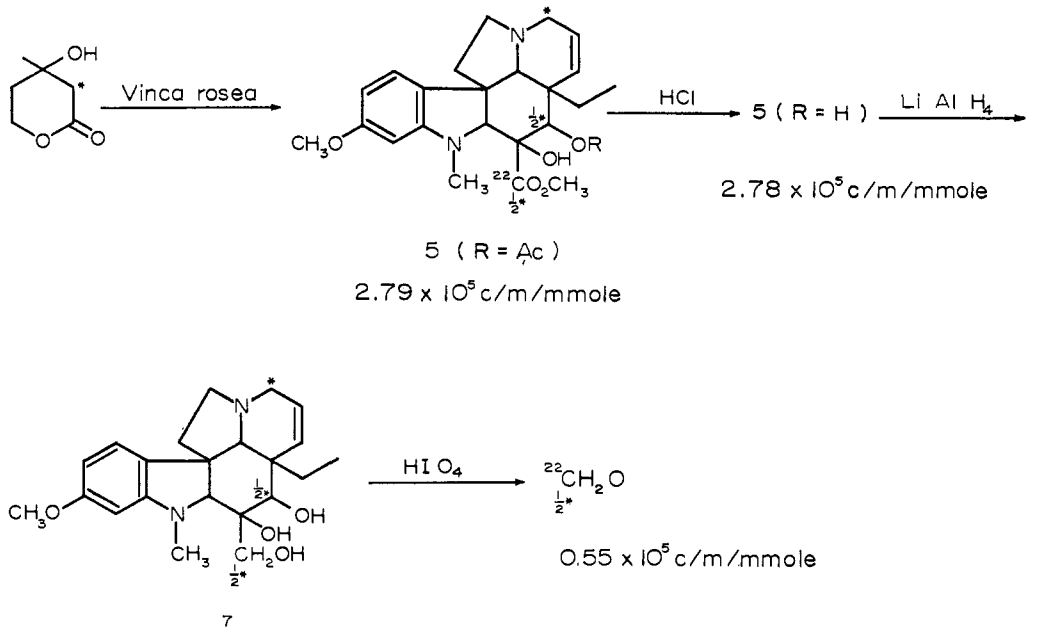


Figure 3.

vindoline (**5**).[26] Accordingly [2-^{14}C]mevalonic acid was administered hydroponically to freshly cut shoots of *Vinca rosea*. After 7 days the plants were harvested and radioactive vindoline isolated by thin layer chromatography. After dilution with authentic vindoline the alkaloid was converted to its hydrochloride and crystallized to constant radioactivity (specific incorporation 0.02%).[1a] According to the monoterpenoid theory (Figure 2) the incorporation of [2-^{14}C]mevalonic acid should produce vindoline (**5**, R = Ac) with the labeling pattern shown (Figure 3), and our subsequent degradative experiments were designed to provide evidence for this prediction. Acid hydrolysis of vindoline (**5**, R = Ac) gave desacetylvindoline (**5**, R = H) with total retention of radioactivity, and we were thus reassured that mevalonic acid had not been degraded to acetate before incorporation. Reduction of desacetylvindoline (**5**, R = H) to vindolinol (**7**) followed by cleavage with periodic acid yielded formaldehyde which was isolated as the crystalline dimedone derivative. Rigorous purification by preparative thin layer chromatography and crystallization from ethanol gave a product with constant radioactivity. This accounted

(26) (a) M. Gorman, N. Neuss, and K. Biemann, *J. Am. Chem. Soc.*, **84**, 1058 (1962); (b) G. H. Svoboda, N. Neuss, and M. Gorman, *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 659 (1959).

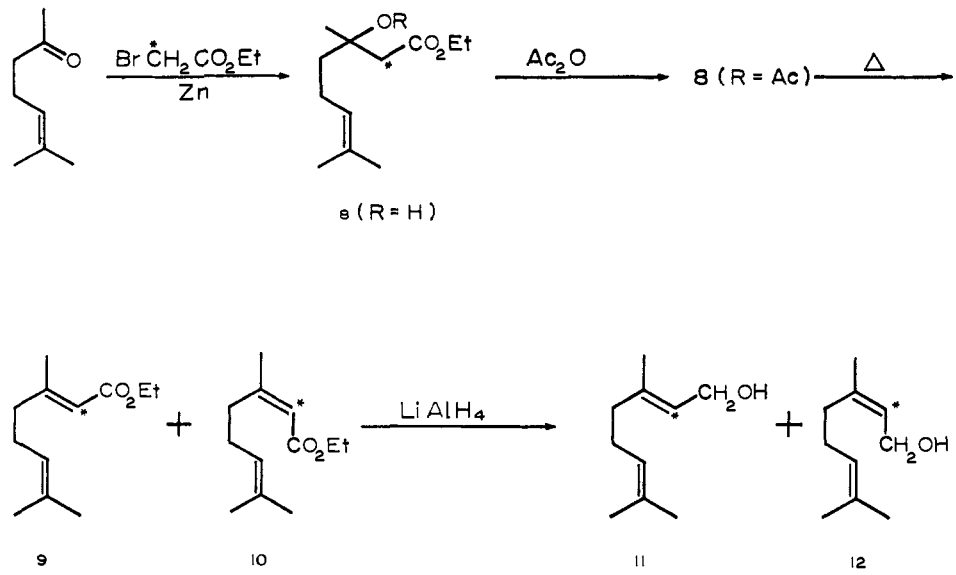


Figure 4.

for 20% of the original activity present in vindoline and thus the proportion of radioactivity located at C_{22} is in reasonable agreement with the value of 25% predicted by the monoterpenoid theory (Figure 2).[16] The positive and specific incorporation of mevalonic acid into the C_{10} unit of vindoline was independently demonstrated by Goeggel and Arigoni[27] who found that 22.5% of the total activity was located at C_{22}. Further support for the terpenoid origin of the C_9–C_{10} unit in the indole alkaloids was provided by Battersby and his coworkers[28] who reported the specific incorporation of mevalonic acid into representative examples of the corynanthe, aspidosperma, and iboga groups of indole alkaloid. In each case the distribution of radioactivity was in full agreement with the biosynthetic proposals made by Wenkert[14c] and Thomas[15] (Figure 2). The simultaneous outcome of all of these studies firmly supported the notion that the C_9–C_{10} unit in the indole alkaloids was terpenoid in origin, and in each case the distribution of radioactivity was in full agreement with proposals made by Wenkert and Thomas.

According to the biogenetic isoprene rule[29] monoterpenes are formed in nature by suitable modification (cyclization–oxidation–reduction) of geranyl, neryl, or linaloyl pyrophosphate. We decided therefore to test the validity of this proposal for the C_9–C_{10} unit of the indole alkaloids. For this purpose [2-^{14}C]geraniol (**11**) was synthesized (Figure 4) from 6-methyl hept-5-en-2-one and methyl [2-^{14}C]bromoacetate using a small-scale modification of the standard route.[30–33] Vapor phase chromatography showed that the final product was a 2:1 mixture of [2-^{14}C]geraniol (**11**) and [2-^{14}C]nerol (**12**). Since no information was available as to the exact nature of the biosynthetic precursor of the monoterpenes we decided to use the product mixture without separating it into its components. The pyrophosphate esters[34] were then synthesized, but before proceeding further we decided to test the precursor activity of the free alcohols. Plant tissue (*e.g.*, *Vinca rosea*) is permeable to geraniol, but at high concentrations we noted a deleterious effect (see Experimental Section). Several feeding experiments were carried out using nonradioactive geraniol to determine optimum feeding conditions. It was found that when geraniol was made water soluble with Tween 20 it could be administered through the cut ends of shoots without obvious damage to the plants. Hydroponic administration of [2-^{14}C]geraniol and [2-^{14}C]nerol (made water soluble by addition of Tween 20) to freshly cut shoots of 1-year-old *Vinca rosea* plants and isolation after 7 days gave radioactive vindoline (**5**, R = Ac) which was purified as its dihydrochloride to constant radioactivity (specific incorporation 0.37%).[1c] Regeneration of the free base and dilution with authentic nonradioactive vindoline yielded a product which, after crystallization from ether, was suitable for degradative experiments. If the monoterpenoid hypothesis is correct [2-^{14}C]geraniol and/or [2-^{14}C]nerol should be incorporated into vindoline (**5**) with the total radioactivity located at position 5 (Figure 5). Kuhn–Roth oxidation of vindoline yielded a mixture of acetic (C_{20}, C_{21}) and propionic (C_{20}, C_{21}, C_5) acids which were separated, purified, and counted as their *p*-bromophenacyl derivatives. In this way it was shown that the propionic acid contained 99% of the total radioactivity present in vindoline while acetic acid was radioinactive. The total radioactivity in vindoline as a result of [2-^{14}C]geraniol and/or [2-^{14}C]nerol incorporated was thus located specifically at position 5. The equivalence of geraniol and nerol as precursors of the C_{10} unit in indole alkaloids has recently been demonstrated by

(27) H. Goeggel and D. Arigoni, *Chem. Commun.*, 538 (1965).
(28) A. R. Battersby, R. T. Brown, R. S. Kapil, A. O. Plunkett, and J. B. Taylor, *ibid.*, 46 (1966).
(29) L. Ruzicka, A. Eschenmoser, and H. Heusser, *Experientia*, **9**, 359 (1953); L. Ruzicka, *Proc. Chem. Soc.*, 341 (1959).
(30) L. Ruzicka and H. Schinz, *Helv. Chim. Acta*, **23**, 959 (1940).
(31) A. Murray and D. L. Williams, "Organic Syntheses with Isotopes," Vol. III, Interscience Publishers, New York, N. Y., 1958, p 650.
(32) A. Vodoz and H. Schinz, *Helv. Chim. Acta*, **33**, 1313 (1950).
(33) H. Farre and H. Schinz, *ibid.*, **35**, 1632 (1952).
(34) F. Cramer and G. Weimann, *Chem. Ind.* (London), 46 (1960); F. Cramer, *Angew. Chem.*, **72**, 236 (1960); F. Cramer and W. Böhm, *ibid.*, **71**, 775 (1959); J. G. Popjak, *et al.*, *J. Biol. Chem.*, **237**, 56 (1962).

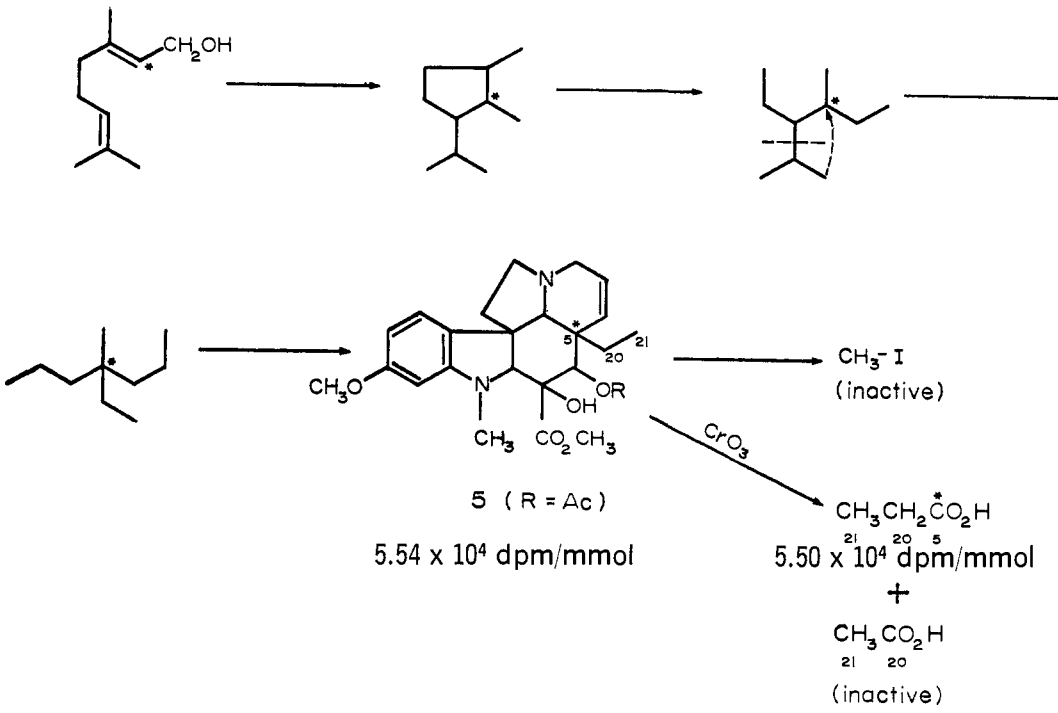
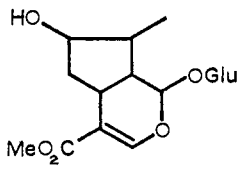


Figure 5.

Battersby and his coworkers.[35] The N-methyl and O-methyl groups were also shown to be nonradioactive, and thus degradation of the precursor and incorporation into the one-carbon pool had not occurred.

This result confirms the monoterpenoid origin of the C_{10} unit in vindoline and in particular indicates that it is constructed and modified in accordance with the biogenetic isoprene rule.[29] Essentially identical results have been obtained by Arigoni,[36] Battersby,[35,37] Leete,[38] and their respective coworkers. Beyond this, Battersby, *et al.*,[39] have reported the successful and specific incorporation of the cyclopentanoid monoterpene loganin (**14**) into the indole alkaloids elaborated by *Vinca rosea*.



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More information is required before a detailed description of indole alkaloid biosynthesis can be presented but the encouraging results of the recent past certainly indicate that before long a complete biosynthetic flow sheet will be available.

Experimental Section

Melting points were determined on a Kofler block and are uncorrected. Ultraviolet spectra were measured on a Cary 14 spectrophotometer in 95% ethanol. The infrared spectra were taken on a Perkin-Elmer Model 137B or a Model 21 spectrophotometer. Alumina G plates or silica gel G plates (according to Stahl) were used for thin layer chromatography. In some cases, the background was made fluorescent to long-wave ultraviolet light by admixture of alumina or silica with 2% GE Ratma p-1, Type 118-2-7 electronic phosphor. Mass spectra were measured on an Atlas CH-4 mass spectrometer and nmr spectra in a Varian A-60 instrument and the line positions were measured with reference to tetramethylsilane as internal standard. Radioactivity was measured with a Nuclear Chicago Model D47 gas flow detector operated as a Geiger counter and mounted in a Model M-5 semiautomatic sample changer in conjunction with a Model 181B Decade Scaler. The activities were measured by depositing samples of 0.2–0.5 mg as thin films on standard aluminum planchettes (1.125 in. diameter). For more accurate determinations, a Nuclear Chicago Model 180040 liquid scintillation counter was used. The scintillation mixture consisted of toluene (500 ml), 2,5-diphenyloxazole (PPO) (2 g), and 2-*p*-phenylenebis(5-phenyloxazole) (POPOP) (25 mg).[40] DL-[2-^{14}C]-Mevalonolactone and ethyl [2-^{14}C]bromoacetate were supplied by Merck Sharp and Dohme of Canada, Ltd.

Administration of DL-[2-^{14}C]Mevalonic Acid to *Vinca rosea* Linn (*Catharanthus roseus* G. Don). DL-[2-^{14}C]Mevalonic acid (7.74 mg; 0.30 mCi) was administered hydroponically to ten freshly cut shoots of *V. rosea*. The plants were continuously illuminated with fluorescent lights for 7 days, water being supplied periodically to keep the stem ends immersed. After 7 days the plants were cut up and dried in a heated vacuum desiccator (60°) for 28 hr. The dried plant material (3.9 g) was macerated in a Waring Blendor with a mixture of methanol (150 ml) and acetic acid (15 ml) and the green solution filtered. Evaporation of the filtrate yielded a crude product which was partitioned between benzene (one 150-ml portion, one 70-ml portion) and 2 *N* hydrochloric acid (one 50-ml portion, four 10-ml portions). The aqueous layers were combined, mixed with an equal volume of chloroform, and filtered through Celite to remove emulsifying material. After further extraction with chloroform (four 25-ml portions) the aqueous layer was made alkaline with ammonia, and the crude alkaloids were extracted into chloroform (six 25-ml portions). Removal of the solvent yielded crude alkaloid (17.6 mg) with a specific activity of 1.77×10^5 counts/(min mg).

Vindoline (5.1 mg; 4,209 counts/(min mg) was isolated[26] from the crude alkaloidal extract by preparative thin layer chromatography (alumina, chloroform–ethyl acetate, 1:1). Dilution with nonradioactive vindoline (11.4 mg) and crystallization from

(35) A. R. Battersby, R. T. Brown, R. S. Kapil, J. A. Knight, J. A. Martin, and A. O. Plunkett, *Chem. Commun.*, 810 (1966).
(36) P. Loew, H. Goeggel, and D. Arigoni, *ibid.*, 347 (1966).
(37) A. R. Battersby, R. T. Brown, J. A. Knight, J. A. Martin, and A. O. Plunkett, *ibid.*, 346 (1966).
(38) E. Leete and S. Ueda, *Tetrahedron Letters*, 4915 (1966).
(39) A. R. Battersby, R. T. Brown, R. S. Kapil, J. A. Martin, and A. O. Plunkett, *Chem. Commun.*, 890 (1966).
(40) E. Rapkin, *Anal. Biochem.*, **1**, 279 (1960).

ether gave vindoline (11.6 mg) with specific activity 620 counts/(min mg) (2.83×10^5 counts/(min mmol). Further crystallization and treatment of an ethereal solution with dry hydrochloric acid produced vindoline hydrochloride which was crystallized from ethyl acetate and methanol–ethyl acetate to constant radioactivity 573 counts/(min mg); 2.82×10^5 counts/(min mmol); specific incorporation 0.02%.

Desacetylvindoline (5, R = H).[41] Vindoline hydrochloride (7.8 mg, 0.0158 mol; specific activity 2.79×10^5 counts/(min mmol)) was heated on a steam bath for 8 min with concentrated hydrochloric acid (0.25 ml). Chloroform, water, and sodium carbonate were added, and after shaking, the organic layer was separated. The aqueous layer was extracted several times with small volumes of chloroform, and the total organic extracts were combined. Renewal of the solvent yielded desacetylvindoline (5.9 mg) which resisted crystallization. Tlc examination (alumina, chloroform–ethyl acetate 1:1) indicated that the product was substantially free from impurity. The specific activity of the noncrystalline material was 2.13×10^5 counts/(min mmol). Further purification by preparative thin layer chromatography (silica gel G, methanol–chloroform 1:20) yielded pure desacetylvindoline (specific activity 2.78×10^5 counts/(min mmol).

Vindolinol (7).[41] Desacetylvindoline (**5**, R = H) (4.45 mg; specific activity 2.78×10^5 counts/(min mmol)) was dissolved in dry tetrahydrofuran (0.5 ml) and a saturated solution of lithium aluminum hydride in ether (3 ml) added. After 4 days in a stoppered reaction tube the mixture was treated with saturated sodium sulfate solution and the ether layer separated. The aqueous layer was extracted repeatedly with chloroform, and the ether and chloroform extracts were combined and dried over sodium sulfate. Removal of the solvent yielded crude vindolinol (4.5 mg) which was used in oxidation experiments.

Periodate Oxidation of Vindolinol (7). Radioactive vindolinol (**7**) (4.5 mg) was dissolved in one drop of alcohol and an aqueous solution of periodic acid (0.13 ml, 0.104 *M*) added. The mixture was allowed to stand with occasional shaking for 2 hr during which time the original precipitate dissolved and the solution darkened considerably. After 2 hr a saturated solution of dimedone (1 ml, 0.0297 *M*) was added and the mixture left overnight at room temperature. The resulting precipitate of dimedone formaldehyde (2.3 mg) was purified by preparative thin layer chromatography (silica gel G, methanol–chloroform 1:50) by elution from the plate with 15% methanol–chloroform. Crystallization from ethanol yielded partially purified material (0.6 mg), mp 165–170° (lit.[42] mp 189°) (0.395×10^5 counts/(min mmol)). The above material was purified further by dissolving in chloroform and extracting with 2% sodium hydroxide. Acidification of the alkaline extract with one drop of concentrated hydrochloric acid yielded crystalline dimedone formaldehyde with specific activity 0.504 counts/(min mmol). Recrystallization to constant radioactivity from ethanol produced pure derivative, mp 192° (0.552×10^5 counts/(min mmol)). This figure represents 20% of the total activity originally found in vindoline.

Ethyl [2-^{14}C]3-Hydroxy-3,7-dimethyl-6-octenoate (8, R = H).[30,31] Ethyl [2-^{14}C]bromoacetate (1.05 g, 6.28 mmol; 0.159 mCi/mmol) was mixed with freshly distilled 6-methyl-5-hepten-2-one (0.794 g, 6.28 mmol) and the mixture slowly added, under nitrogen, to a 10% excess of activated granular zinc (0.452 g). The reaction commenced after brief warming when about 10% of the reagent mixture had been added. Dropwise addition was then continued at such a rate as to maintain reflux in the stirred reaction mixture. After refluxing for 1 hr more the reaction was cooled in an ice bath and treated with ice-cold 10% sulfuric acid (5 ml). The resulting solution was extracted with benzene (two 5-ml portions) and ether (two 5-ml portions), and the combined organic extracts were washed and dried. Removal of the solvent yielded the crude product which was distilled in a sublimation apparatus modified by addition of a small glass cup (2 ml) at the end of the cold finger. Sufficiently pure ester **8** (R = H) was thus obtained (980 mg, 4.56 mmol; 72.5%) with spectroscopic properties in agreement with this structure: ν_{max} (film) 3540 (s), 3000 (s), 2940 (s), 1640 (s), 1460 (m), 1380 (m), 1330 (m), 1205 (s), 1040 (m), 935 (w), 840 cm^{-1}; τ ($CDCl_3$) 4.89 (broad triplet, 1 H), 5.89 (quartet, 2 H), 6.64 (singlet, 1 H), 7.56 (singlet, 2 H), 7.99 (multiplet, 2 H), 8.33 and 8.39 (singlets, 6 H), 8.77 (singlet, 3 H), and 8.77 (triplet, 3 H).

Ethyl [2-^{14}C]3-Acetyl-3,7-dimethyl-6-octenoate (8, R = Ac).[32] The hydroxy ester **8** (R = H) (980 mg, 4.56 mmol) was refluxed for 6.5 hr with a 2 *M* excess of acetic anhydride. After treatment with water (5 ml) to decompose residual acetic anhydride the reaction mixture was extracted with ether (three 5-ml portions). The organic extracts were washed free of acid, dried, and evaporated to yield impure acetate **8** (R = Ac) (1.1 g, 4.34 mmol; 95% yield) whose infrared spectrum showed that the band at 3540 cm^{-1} was absent and that new bands at 1740 and 1245 cm^{-1} had appeared.

***cis*- and *trans*-Ethyl [2-^{14}C]3,7-Dimethyl-2,6-octadienoates (9 and 10).**[32] Ethyl [2-^{14}C]3-acetyl-3,7-dimethyl-6-octenoate (1.11 g, 4.35 mmol) was slowly distilled at atmospheric pressure into the collection cup of a microdistillation apparatus. Distillate and residue were then dissolved in ether and the solution washed with 10% sodium carbonate solution (three 3-ml portions), water (four 4-ml portions), and brine (2 ml). Work-up in the usual way gave a crude oil which was distilled under reduced pressure (16 mm). The product was a mixture (1:3) of *cis*-ethyl 3,7-dimethyl-2,6-octadienoate (**10**) and its *trans* isomer **9** (0.70 g, 3.58 mmol; 83% yield). The composition and identity of this product were made possible by exhaustive studies with nonradioactive material. As expected the infrared bands at 1735 and 1250 cm^{-1} had disappeared and a new band was noted at 1650 cm^{-1}. In addition the nmr spectrum had a singlet at τ 4.38 (olefinic proton on newly formed double bond, area 0.90 H).

Pyrolysis studies with nonradioactive substrate were monitored by vapor phase chromatography using a Carbowax 20M (5 ft × 0.25 in.) column at 140°. Collector, detector, and injector temperatures of 150, 250, and 235°, respectively, were used, with a helium flow rate of 59 cc/min. Separation of the *cis* and *trans* isomers was accomplished with a Carbowax 20M (10 ft × 0.318 in.) column at 180° and a helium flow rate of 100 cc/min. Up to 100 μl of material could be separated under these conditions.

[2-^{14}C]3,7-Dimethyl-2,6-octadien-1-ol ([2-^{14}C]Geraniol) (11).[33] Ethyl [2-^{14}C]3,7-dimethyl-2,6-octadienoate (0.70 g, 3.58 mmol; 3:1 mixture of **9** and **10**) in ether (3 ml) was added to a stirred ethereal solution (5 ml) of lithium aluminum hydride (100% excess) at such a rate as to maintain reflux. The mixture was then refluxed for 40 min and excess hydride destroyed by cautious addition of wet ether. After treatment with dilute hydrochloric acid (3 ml, 2 *N*)) the ether layer was separated and the aqueous layer extracted further with ether (three 3-ml portions). The combined organic extracts were washed with water (five 3-ml portions) and brine (2 ml) and dried over magnesium sulfate. Removal of the solvent under nitrogen yielded a crude product which was distilled *in vacuo* (12 mm) to provide a mixture of [2-^{14}C]geraniol (**11**) and [2-^{14}-C]nerol (**12**), yield 0.3736 g, 2.46 mmol, 68%. The infrared spectrum was identical with that from the nonradioactive synthesis.

Administration of [2-^{14}C]Geraniol–[2-^{14}C]Nerol to *Vinca rosea* Linn (*Catharanthus roseus* G. Don). The plants (1 year old) were grown in an unheated, shaded greenhouse and about 5% were flowering. The plants had flowered the previous summer and had no flowers for 3 months prior to administration of geraniol. The crude alkaloid content based on dried weight of stem and leaf material was 0.37% and vindoline was shown to be present by thin layer chromatography (crimson color with ceric sulfate spray; 1% ceric sulfate–35% sulfuric acid).[26b,41]

Assimilation of geraniol by the plant was confirmed in the following manner. A 6-in. cutting (21 leaves) was placed in a test tube containing an aqueous suspension (1 ml) of [2-^{14}C]3,7-dimethyl-2,6-octadien-1-ol (1.67 mg, 0.159 mCi/mmol). The suspension was prepared by shaking the labeled precursor with a solution (1 ml) of one drop of Tween 20 in distilled water (250 ml). Within 3 days activity was detectable with radiation monitor throughout the plant. This was also confirmed by radioautographs which were obtained by leaving the stem and leaves in carrier with Ilford X-ray film for 7 days.

The toxicity of geraniol as a functionary concentration was checked in a simple experiment by administration of 1 ml of solution containing the following concentrations of geraniol and Tween 20, respectively: 1 mg, $^1/_{2500}$ drop; 1 mg, $^1/_{10}$ drop; 2 mg, $^1/_{2500}$ drop; 2 mg, $^1/_{10}$ drop; 5 mg, $^1/_{10}$ drop. It was concluded by visual inspection that healthy cuttings of *Vinca rosea* will tolerate up to $^1/_{10}$ drop of Tween 20 and 2 mg of geraniol in 1 ml of water.

After preliminary successful incorporation experiments, the following preparative scale procedure was used. [2-^{14}C]Geraniol (0.272 mg; specific activity 0.159 mCi/mmol, 0.282 mCi) was emulsified in distilled water (200 ml) with Tween 20 (eight drops) and administered to the cut ends of 192 *Vinca rosea* cuttings over a period of 7 days. Constant illumination (fluorescent tubes) was

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provided and care was taken to ensure that the cut ends were fully immersed.

After 7 days the fresh plant material (net weight was 835 mg) was macerated (in 50-g portions) with methanol–acetic acid (10:1; three 200-ml portions) in a Waring Blendor. The solvent was removed and the residue partitioned between benzene and 2 *N* hydrochloric acid. The combined aqueous layer was extracted further with chloroform treated with ammonium hydride (pH 10) and the alkaloid extracted into chloroform. In this way a yield of 2.14 g of crude alkaloid was obtained (0.26%).

Vindoline was separated from the crude alkaloidal fraction by preparative thin layer chromatography. Six chromatoplates were prepared (Desaga apparatus: blade setting 0.8 mm) by spreading a slurry of alumina G (50 g) and phosphor (1 g) in water (90 ml) on each 20 × 60 cm plate. The crude alkaloid was applied to the plates which were developed in chloroform–ethyl acetate (1:1, 8 hr), and the vindoline-containing bands (R_f 0.3–0.4) were located by reference to qualitative plates. The relevant bands were scraped from the plates immediately after development and eluted successively with chloroform and methanol.

Tlc examination of the crude alkaloid (73 mg) obtained in this way showed the presence of other materials besides vindoline, and further purification (in the manner described above but using 20 × 20 cm plates) was necessary before a reasonably pure sample of vindoline was obtained (20 mg, 1350 counts/(min mg), rate of incorporation 0.011%). The ultraviolet spectrum, λ_{max} 252 and 303 mμ (ϵ_{max} 7600 and 5500), was identical with that of authentic vindoline, as was its chromatographic behavior (crimson with 1% ceric sulfate–35% sulfuric acid spray[26b]).

Further purification was achieved by converting vindoline to its dihydrochloride and crystallization of the latter from methanol–ethyl acetate. The dihydrochloride so obtained was counted on aluminum planchettes (940 counts/(min mg), 5.0 × 10^5 counts/(min mmol)). Repeated recrystallization yielded pure material, mp 150–152° (960 counts/(min mg), 5 × 10^5 counts/(min mmol)). The free alkaloid was regenerated by shaking a chloroform solution (4 ml) of the salt with 1 *N* ammonium hydroxide, and the reconverted vindoline (6.27 mg)) showed no fluorescent impurities on thin-layer chromatographic plates (5.1 × 10^5 counts/(min mmol), 1120 counts/(min mg)). Constant activity had thus been achieved and the specific incorporation of the [2-^{14}C]geraniol–[2-^{14}C]nerol mixture into vindoline was 0.37%.

The active vindoline (6.27 mg) obtained above was diluted with authentic vindoline (111.6 mg) and crystallized from ether. The level of radioactivity in the diluted vindoline was measured in toluene scintillation mixture[40] using a Nuclear-Chicago Model 180040 liquid scintillation counter. The counting efficiency was established by the channel ratio method and the specific activity calculated to be 5.54 × 10^4 dpm/mmol.

Oxidation of [^{14}C]Vindoline (5).[43-45] A series of 13 experiments were required to establish the oxidation conditions under which a suitable ratio of propionic and acetic acids could be obtained so that the *p*-bromophenacyl esters of these acids could be purified and their specific activities determined. The largest scale experiment is described below.

[^{14}C]Vindoline (93.6 mg, 0.205 mmol; 5.54 × 10^4 dpm/mmol) was added to 30% aqueous chromium trioxide (5 ml)[43] and the mixture distilled. Fractions (60 ml) of steam-volatile distillate were collected while keeping the volume of the oxidation mixture constant (~5 ml). Each fraction was titrated with carbonate-free lithium hydroxide (0.0128 *N*), and the total quantity of volatile acid obtained from oxidation of vindoline was calculated (1.97 equiv). The fractions were then evapoated *in vacuo* (55°) to small volume (~0.5 ml) and treated with ethanol (10 ml). *p*-Bromophenacyl bromide (125 mg, 0.48 mmol; 10% excess) was added, and the solution was refluxed for 40 min. The product obtained was examined by thin layer chromatography (silica gel G with fluorescent background; 0.5 mm × 20 cm × 20 cm plates) and six bands (A–H) were removed and eluted with ether. Bands B (R_f 0.12–0.25, 40 mg) and D (R_f 0.26–0.30, 4 mg) were shown by subsequent tlc examination to be *p*-bromophenacyl acetate (R_f 0.37) and *p*-bromophenacyl propionate (R_f 0.53), respectively. Further purification of the propionate derivative by tlc (0.1 mm × 5 cm × 20 cm silica gel plate) and crystallization (three times) from petroleum ether (bp 40–60°) yielded pure *p*-bromophenacyl propionate, mp 59–60° (lit.[46] mp 61–62°), which was counted in the toluene scintillation mixture (specific activity 5.50 × 10^4 dpm/mmol, *i.e.*, 99.3 ± 2% of original vindoline activity).

In a similar fashion *p*-bromophenacyl acetate was purified and crystallized (three times) from petroleum ether (bp 30–60°), mp 83–84° (lit.[46] mp 84–85°). No activity could be detected in this compound (24 ± 0.5 counts/min; background 24 ± 1 counts/min).

Estimation of N-Methyl and O-Methyl Groups[47] in Vindoline (5, R = Ac). An alkoxyl determination on nonradioactive vindoline was accomplished using literature methods,[47] and it was shown that 2.87 equiv of methyl iodide was produced. Active vindoline (4.197 mg, specific activity 5.54 × 10^4 counts/(min mmol)) was then treated similarly with hydriodic acid and the methyl iodide produced was added directly to the scintillation mixture (5 ml). No activity above background could be detected in the methyl iodide.

Acknowledgments. We thank Dr. C. T. Beer (Cancer Research Center, University of British Columbia) for the gift of *C. roseus* plants and for advice on cultivation. We are also grateful to Dr. M. Gorman and Dr. N. Neuss (Eli Lilly Co.) for providing samples of vindoline and other alkaloids. It is a pleasure to acknowledge the assistance of Mr. P. Salisbury of this department during the course of this work. This investigation was supported by generous financial assistance from the National Research Council (NRC 2267, 1480).

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