INCORPORATION OF [2-^{14}C, 2',3'-$^{13}C_2$]TRYPTOPHAN INTO VINDOLINE ESTABLISHED BY ^{13}C NMR

EDWARD LEETE

Natural Products Laboratory,[1] *School of Chemistry, University of Minnesota, Minneapolis, MN 55455*

ABSTRACT.—DL-[2-^{14}C, 2',3'-$^{13}C_2$]Tryptophan was prepared from [2-^{14}C]indole, [^{13}C]formaldehyde, and diethyl [2-^{13}C]malonate. This amino acid, labeled with contiguous ^{13}C atoms in its side chain, was fed to the plant *Catharanthus roseus*, resulting in the formation of radioactive vindoline (0.19% specific incorporation). An examination of the ^{13}C nmr spectrum of this alkaloid revealed the presence of satellites located about the signal of C-6, which were due to spin-spin coupling of the contiguous ^{13}C atoms located at C-5 and C-6 of vindoline. Since the specific incorporation, deduced from the intensity of the satellites relative to the central singlet peak, was the same as that of the ^{14}C located in the indole nucleus, the intact incorporation of tryptophan into vindoline is unequivocally established.

Most of the early work on the biosynthesis of secondary natural products involved the use of putative precursors labeled with ^{14}C, the radioactive isotope of carbon. Often quite lengthy and complicated degradations had to be carried out to determine the location of the ^{14}C in the ultimate natural product. It is now possible to avoid these degradations by the use of ^{13}C as a tracer. This isotope is not radioactive, however, its location in a natural product, derived from a precursor which is enriched with ^{13}C above the natural abundance (1.11%) is possible through the use of ^{13}C nmr. The use of precursors labeled with a single ^{13}C atom has been used extensively in the study of the biosynthesis of microbial natural products (1–3), since one can usually expect high specific incorporations[2] in such systems. The labeled position in the natural product will have an enhanced signal at that carbon in its ^{13}C nmr spectrum. Since the intensity of nmr signals is somewhat variable, this method can only be used when the enhancement of the signal above the natural abundance is at least 30–40%. For example, a natural product was found to have 1.6% ^{13}C at a certain position, and was derived from a precursor enriched 90% at the analogous carbon. The specific incorporation would be 0.55%, and the nmr signal for this carbon would be enhanced 45%. In higher plants the specific incorporation of precursors into alkaloids and other natural products is usually quite low, and only a few examples using this method have been reported (4–9).

A much greater dilution of the administered precursor can be tolerated if one labels with contiguous ^{13}C atoms. The natural occurrence of such contiguous carbons is only 1.11% x 1.11% = 0.0123%. Furthermore, these contiguous carbon-13 atoms give rise to satellites (due to spin-spin coupling) in the ^{13}C nmr, located about the central singlet peaks, which arise from natural abundance ^{13}C and the incorporation of singly labeled species. This method was first used in biosynthetic studies by Seto and coworkers (10, 11), who fed [1,2-$^{13}C_2$]acetic acid to various microbial systems. We have used [5,6-$^{13}C_2$]nicotinic acid for investigating the biosynthesis of the tobacco alkaloids (12) and dioscorine (13).

---

[1]Contribution No. 170 from this laboratory. Part of this work was presented at the annual meeting of the American Society of Pharmacognosy, held at Purdue University, West Lafayette, Indiana, July 29-August 3, 1979.

[2]Specific incorporation = % excess ^{13}C in the natural product/% excess ^{13}C in the administered precursor. For radioactive compounds: dpm/mM of the natural product/dpm/mM in the precursor.

It has also been shown that [2,3-$^{13}C_2$]ornithine yields nicotine and nornicotine which are labeled symmetrically with contiguous ^{13}C atoms on either side of their pyrrolidine rings (14).

In the present article, we describe the synthesis of tryptophan labeled with contiguous ^{13}C atoms in its side chain, and we have demonstrated its utility for investigating the biosynthesis of the indole alkaloids found in *Catharanthus roseus*. The synthesis of DL-[2-^{14}, 2',3'-$^{13}C_2$]tryptophan (**5**) is illustrated in figure 1, and

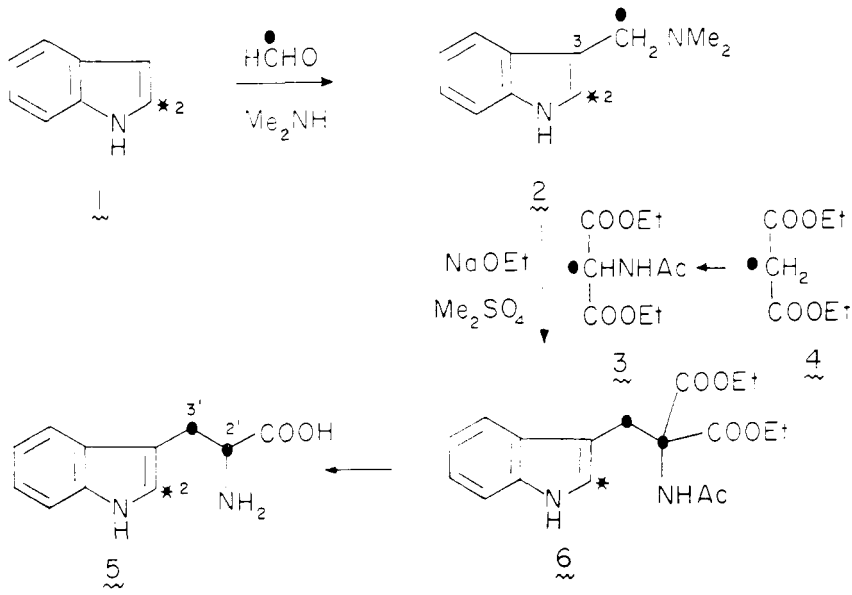

FIG. 1. Synthesis of DL-[2-^{14}C, 2',3'-$^{13}C_2$]Tryptophan. (* ^{14}C, ● ^{13}C)

is based on the previously described preparation of unlabeled tryptophan (see Experimental). The amino acid was labeled with ^{14}C at C-2 of its indole nucleus so that its specific incorporation into the ultimate alkaloids, and the rate of uptake into the plants could be readily determined by radioactive assay. The ^{13}C nmr spectrum of the ^{13}C-enriched tryptophan is illustrated in figure 2. The chemical shifts were assigned by comparison with model compounds: indole and 3-methylindole (15). Our observed values were in good agreement with the previously published ^{13}C nmr spectra of natural abundance tryptophan (16–18). The enriched positions, C-2' and C-3', exhibit the expected satellites with a coupling constant of 33.2 Hz. The satellites are not symmetrically located about the central peaks. This is due to an approach to an AB spin system (19). The unenriched carbons which are adjacent to C-2' and C-3' were also split into triplets: $^1J_{1'2'} = 53.5$ Hz, $^1J_{3,3'} = 44$ Hz.

The labeled tryptophan was fed to *Catharanthus roseus* plants by the wick method. In order not to overload the plants with tryptophan, the feeding was spread over 5 days. The plants were harvested after two weeks, and the alkaloids isolated and separated as previously described (20). Vindoline (see figure 3) is the most abundant alkaloid in the plant, and radioactive assay indicated that the specific incorporation of tryptophan into this alkaloid was 0.19%. Its ^{13}C nmr spectrum has been previously determined (21), and we obtained essentially

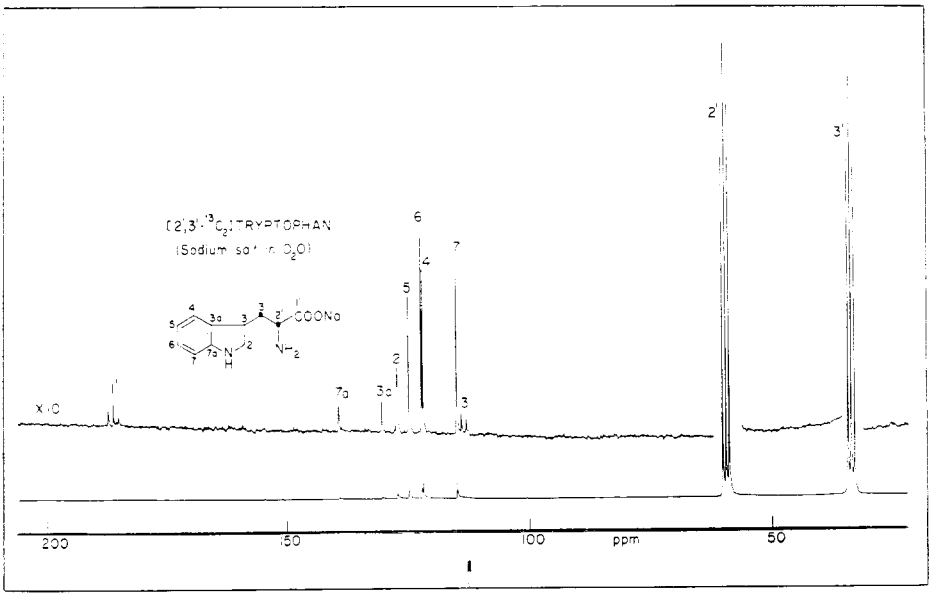

FIG. 2. Proton noise-decoupled FT^{13}C NMR spectrum of [2',3'-$^{13}C_2$]tryptophan in D_2O as its sodium salt. The upper spectrum is enhanced ten times in the vertical scale, so that the natural abundance carbons can be observed.

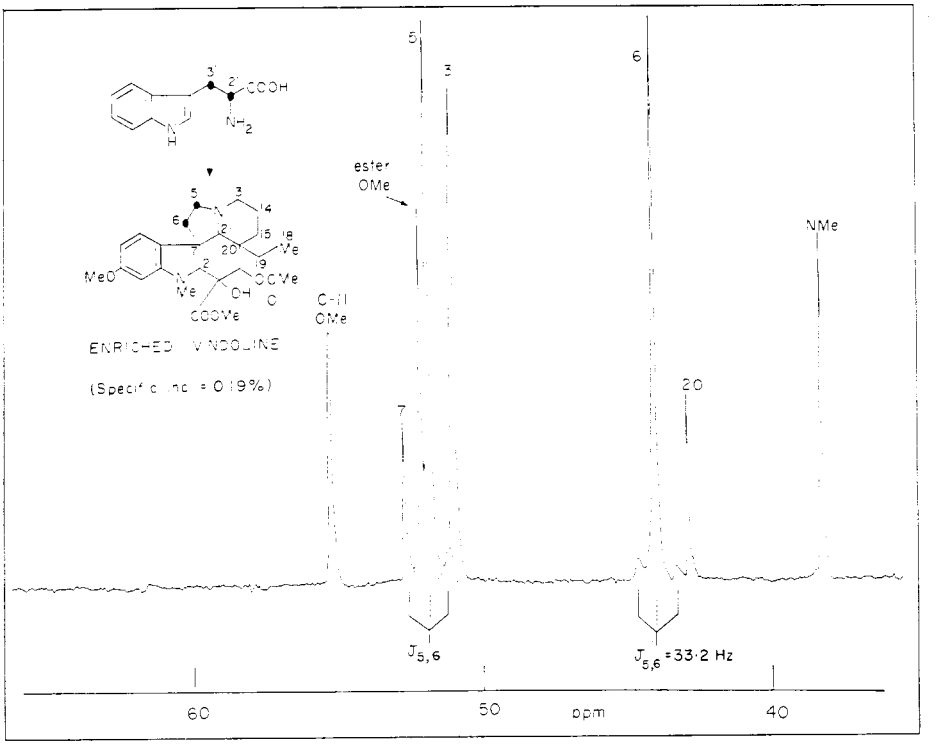

FIG. 3. Proton noise-decoupled FT ^{13}C NMR spectrum of enriched vindoline (aliphatic region) in $CDCl_3$.

the same spectrum except that we found that C-5 (51.9 ppm from Me_4Si) was resolved from the ester OMe group (52.1 ppm). The previous work assigned both these carbons to a signal at 51.9 ppm. The carbons which are expected to be enriched in the vindoline are C-5 and C-6. Figure 3 illustrates that region of the ^{13}C spectrum of the enriched vindoline where these carbons resonate. Satellites are readily observable at C-6 ($^1J_{5,6}=33.2$ Hz), again asymmetrically arranged in accord with theory. The specific incorporation of ^{13}C into vindoline was determined by measuring the intensity of these satellites relative to the central peak, the calculation taking into account the contribution of singly labeled species to the central peak (22). The satellites at C-5 are not observed since they fall under the signals for C-7 and C-3. However, it is possible to detect shoulders on these signals due to the presence of the satellite peaks.

The specific incorporation of tryptophan into vindoline determined from its ^{13}C nmr spectrum was found to be 0.21%, in excellent agreement with the result obtained by radioactive assay. Since the tryptophan was labeled with ^{14}C in the indole nucleus, and with ^{13}C in the side chain, the present results clearly demonstrate, for the first time, the incorporation of tryptophan as an intact unit into the indole alkaloid vindoline. Previous results (20, 23) only showed that the tryptophan side chain was incorporated into vindoline.

It should be pointed out that this method of studying the biosynthesis of natural products, using ^{13}C labeled precursors, can yield information very rapidly. In the present case, the whole experiment, including feeding, isolation of the vindoline, and determination of its ^{13}C nmr spectrum, was completed in less than a month. Using radioactive tracers it took over a year to establish that [2'-^{14}C] tryptophan yielded vindoline labeled at C-5 (20). A disadvantage of this method of elucidating biosynthetic pathways is that one needs relatively large amounts of the ^{13}C-labeled natural product for determination of its ^{13}C nmr spectrum, especially when the specific incorporation is low. However, we can hope that improvements in instrumentation will make this less of a problem in the future.

EXPERIMENTAL[3]

DL-[2-^{14}C, 2',3'-$^{13}C_2$]TRYPTOPHAN (**5**).—[2-^{14}C]Indole (**1**) (25), dimethylamine, and [^{13}C] formaldehyde (Merck Sharpe and Dohme of Canada) were condensed in dilute acetic acid according to the literature method (26) to afford [2-^{14}C, *methylene*-^{13}C]gramine (**2**). Its proton noise-decoupled ^{13}C nmr spectrum (in dimethylsulfoxide-d_6) was essentially the same as that recorded by Wenkert *et al.* (27), except that the signal at 111.6 ppm (C-3) was split into a triplet by the adjacent ^{13}C enriched methylene carbon ($^1J=54$ Hz). Carbon-2 (124.2 ppm) was also split due to a geminal coupling with the enriched carbon ($^2J=4.8$ Hz). No splitting of the N-methyl groups at 44.7 ppm was observed. Mass spectrometry indicated that the gramine was enriched 71% with ^{13}C. Diethyl [2-^{13}C]malonate (**4**) (Merck Sharpe and Dohme of Canada) was converted to ethyl [2-^{13}C]acetamidomalonate (**3**) by reaction with nitrous acid, followed by reduction of the resultant nitroso compound, dissolved in acetic anhydride, with hydrogen in the presence of platinum on charcoal (28). Mass spectrometry indicated a ^{13}C enrichment of 57% in (**3**). The labeled gramine and (**3**) were condensed in absolute ethanol in the presence of sodium ethoxide and dimethyl sulfate (29) to yield ethyl α-carboethoxy-α-acetamido-β-(3-indolyl)propanoate (**6**). DL-[2-^{14}C, 2',3'-$^{13}C_2$]Tryptophan acetate was obtained from this ester as previously described (28). Since tryptophan does not yield a substantial molecular ion in the mass spectrometer, the distribution of the ^{13}C labeled species was deduced by mass spectrometery on the ester (**6**) which affords a good molecular ion with no $M^+ - 1$ peak. This material was found to contain 40% $^{13}C_2$, 47% $^{13}C_1$ and 13% unenriched. Since the ^{13}C enrichment of the gramine and ethyl acetamidomalonate were 71 and 57% respec-

[3]The ^{13}C nmr spectra were determined by Dr. Robert M. Riddle on a Varian XL-100-15 spectrometer (25.2 MHz) equipped with a VFT-100 Fourier-transform accessory. Mass spectra were determined by Dr. Roger Upham on an AEI-30 spectrometer. Radioactive materials were assayed in duplicate in a Nuclear Chicago Mark II liquid scintillation counter using dioxane-ethanol as solvent with the usual scintillators (24).

tively, the composition of the $^{13}C_1$ species are calculated to be: 16.5% [2'-^{13}C]- and 30.5% [3'-^{13}C]tryptophan.

ADMINISTRATION OF THE DL-[2-^{14}C, 2',3'-$^{13}C_2$]TRYPTOPHAN TO *Catharanthus roseus* AND ISOLATION OF THE ALKALOIDS.—The plants were growing in soil in a greenhouse and were three months old at the time of feeding (July). The plants were grown from seeds purchased at the local supermarket labeled: Vinca (Periwinkle), mixed colors. The DL-[2-^{14}C, 2',3'-$^{13}C_2$]tryptophan acetate (0.33 mM, 4.76 x 10^6 dpm/mM) was fed to 20 plants via cotton wicks inserted into the stems of the plants near to ground level. The feeding was carried out over 5 days, on each day an additional quantity of the tryptophan acetate dissolved in water was added to the beakers in which the wicks were placed. Thus each plant received 3.3 μM of tryptophan per day. Two weeks after the initial feeding, the plants (fresh weight 2.3 kg) were harvested. The residual activity in the beakers was 4.5 x 10^3 dpm (0.3% of the total amount fed). The alkaloids were extracted and separated as previously described (20). Vindoline was finally purified by tlc on Silica gel PF-254 (Merck) developing with a mixture of chloroform, benzene, methanol, and ammonium hydroxide solution (100:200:30:1). The zone (R_f 0.6) corresponding to vindoline was extracted with chloroform, affording after crystallization from ether, colorless needles of vindoline (75 mg), 9.1 x 10^3 dpm/mM (0.19% specific incorporation). Catharanthine (12 mg), 8.3 x 10^3 dpm/mM (0.17% specific incorporation) was also isolated, but the amount was insufficient for observation of satellites in its ^{13}C nmr.

DETERMINATION OF THE ^{13}C NMR SPECTRA.—The DL-[2-^{14}C, 2',3'-$^{13}C_2$]tryptophan (64 mg in 0.4 ml of D_2O, containing an equivalent amount of NaOH, in a 5 mm tube) was run for 13 K transients, 0.73 sec acquisition time, 1.47 Hz/data point. The chemical shifts, ppm from Me_4Si (carbon No.) were: 186.3 (1'), 60.3 (2'), 34.5 (3'), 127.8 (2), 114.5 (3), 131.0 (3a), 122.4 (4), 125.3 (5), 122.7 (6), 115.4 (7), 139.8 (7a). The average distance of the inner satellites to the central peaks of C-2' and C-3' was 16.1 Hz. The theoretical distance, calculated from the formula: $\frac{1}{2}(V_{AB}+J_{AB}-\sqrt{V_{AB}^2+J_{AB}^2})$ (30) where V_{AB}=difference in chemical shift between the coupled carbons (651 Hz for C-2' and C-3'), and J_{AB}=the coupling constant (33.2 Hz for C-2' and C-3'), was 16.2 Hz. The vindoline (72 mg in 0.4 ml of $CDCl_3$ in a 5 mm tube) was run for 20 K transients, 1.6 sec acquisition time, 0.7 Hz/data point. The inner satellite of C-6 was 15.4 Hz from the central peak, in excellent agreement with the calculated value (15.3 Hz), where $V_{5,6}$=199 Hz, and $J_{5,6}$=33.2 Hz.

ACKNOWLEDGMENTS

This investigation was supported by Research Grant GM-13246-22 from the National Institutes of Health, U. S. Public Health Service. I thank Dr. M. Gorman of the Eli Lilly Co. for generous supplies of vindoline and other alkaloids of *Catharanthus roseus*.

Received 30 July 1979.

LITERATURE CITED

1. M. Tanabe, *Biosynthesis*, **2**, 241 (1973); **3**, 247 (1975); **4**, 204 (1976).
2. U. Sequin and A. I. Scott, *Science*, **186**, 101 (1974).
3. A. G. McInnes, J. A. Walter, J. L. C. Wright and L. C. Vining, "Topics in Carbon-13 NMR Spectroscopy", Editor: G. C. Levy, Vol. 2, p. 123, Wiley-Interscience, New York, 1976.
4. C. R. Hutchinson, A. H. Heckendorf, P. E. Daddona, E. Hagaman and E. Wenkert, *J. Am. Chem. Soc.*, **96**, 5609 (1974), **97**, 1988 (1975).
5. A. R. Battersby, P. W. Sheldrake and J. A. Milner, *Tetrahedron Lett.*, 3315 (1974).
6. E. Leete and G. B. Bodem, *J. Am. Chem. Soc.*, **98**, 6321 (1976).
7. E. Leete, K. C. Ranbom and R. M. Riddle, *Phytochemistry*, **18**, 75 (1979).
8. N. Takao, K. Iwasa, M. Kamijauchi and M. Sugiura, *Chem. Pharm. Bull.*, **24**, 2859 (1976).
9. A. Yagi, G. Nonaka, S. Nakayama and I. Nishioka, *Phytochemistry*, **16**, 1197 (1977).
10. H. Seto, T. Sato and H. Yonehara, *J. Am. Chem. Soc.*, **95**, 8461 (1973).
11. H. Seto, W. Cary and M. Tanabe, *J. Chem. Soc. Chem. Comm.*, 867 (1973).
12. E. Leete, *Bioorg. Chem.*, **6**, 273 (1977).
13. E. Leete, *Phytochemistry*, **16**, 1705 (1977).
14. E. Leete and M.-L. Yu, *Phytochemistry*, in press.
15. J. F. Johnson and W. C. Jankowski, "Carbon-13 NMR Spectra", John Wiley and Sons, New York, NY (1972), Spectra No. 283 and 341.
16. J. H. Bradbury and R. S. Norton, *Biochim. Biophys. Acta*, **328**, 10 (1973).
17. A. Allerhand, R. F. Childers and E. Oldfield, *Biochemistry*, **12**, 1335 (1973).
18. K. Voelter, S. Fuchs, R. H. Steuffer and K. Zech, *Monatsh. Chem.*, **105**, 1110 (1974).
19. M. L. Casey, R. C. Paulick and H. W. Whitlock, *J. Am. Chem. Soc.*, **98**, 2636 (1976).
20. E. Leete, A. Ahmad and I. Kompis, *J. Am. Chem. Soc.*, **87**, 4168 (1965).
21. E. Wenkert, D. W. Cochran, F. W. Hagaman, F. M. Schell, N. Neuss, A. S. Katner, P. Potier, C. Kan, M. Plat, M. Koch, H. Mehri, J. Poisson, N. Kunesch and Y. Rolland, *J. Am. Chem. Soc.*, **95**, 4990 (1973).

22. E. Leete, N. Kowanko, R. A. Newmark, L. C. Vining, A. G. McInnes and J. L. C. Wright, *Tetrahedron Lett.*, 4103 (1975).
23. D. Gröger, K. Stolle and K. Mothes, *Tetrahedron Lett.*, 2579 (1964).
24. A. R. Friedman and E. Leete, *J. Am. Chem. Soc.*, **85**, 2141 (1963).
25. E. Leete and L. Marion, *Can. J. Chem.*, **31**, 1195 (1953).
26. H. Kühn and O. Stein, *Chem. Ber.*, **70**, 567 (1937).
27. E. Wenkert, J. S. Bindra, C.-J. Chang, D. W. Cochran and F. M. Schell, *Acc. Chem. Res.*, **7**, 46 (1974).
28. H. R. Snyder and C. W. Smith, *J. Am. Chem. Soc.*, **66**, 350 (1944).
29. N. F. Anderson, S. Archer and C. M. Suter, *J. Am. Chem. Soc.*, **67**, 36 (1945).
30. L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd Edition, Pergamon Press, 1969, p. 129.