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Indole Alkaloid Biosynthesis

An Investigation into Leucine as a Possible Precursor of the Monoterpene Portion of Vindoline

DONALD C. WIGFIELD AND BETTY P. WEN[1]

Department of Chemistry, Carleton University, Ottawa, Canada K1S 5B6.

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Because of the nonspecific incorporation of acetate into the monoterpenoid portion of the indole alkaloids, the amino acid leucine has been investigated as an alternative precursor. Incorporations of leucine into the alkaloids vindoline and catharanthine in *Vinca rosea* are found to be 0.07 and 0.02%, respectively, levels comparable with those of acetate. An equation is developed to calculate the quantity of [^{13}C]leucine needed to be fed in order to obtain alkaloids with sufficient ^{13}C enrichment to be analysed by ^{13}C nmr spectroscopy. This equation assumes that percentage incorporations do not decrease with increasing quantities of leucine fed, an assumption found to be true. [2-^{13}C]Leucine was synthesized from [2-^{13}C]acetic acid in an eight-step procedure in 11.5% overall yield, and fed to *Vinca rosea* plants. Incorporations into vindoline obtained using both ^{14}C and ^{13}C were in reasonable agreement, and the activity was found to be spread over seven carbon atoms, none of which corresponded to the two atoms at which activity was expected. It is concluded that the leucine → mevalonate → monoterpene route does not operate in indole alkaloid biosynthesis, leaving open the question of the origin of the monoterpene portion of the alkaloids. An attempt to confirm these results by degradation of ^{14}C-labelled alkaloids was unsuccessful, vindoline proving to be unexpectedly resistant to oxidation.

INTRODUCTION

Despite decades of ingenious speculation, the biosynthetic origin of the nontryptophan portion of the indole alkaloids remained unknown until 1965, when successful incorporations of labelled mevalonate and geraniol were demonstrated. During the next five years, a flurry of activity followed, from which the entire biosynthetic route from mevalonate to the Corynanthe, the Iboga, and the Aspidosperma alkaloids was deduced. The experiments performed and the biosynthetic route now believed to operate have been summarized by Scott (*1*), whose own contributions included the brilliant use of germinating seedlings which at one shot provided the identity of all the key intermediates in the later stages of the biosynthetic pathway.

Facts do not cease to exist, however, because they are ignored (*2*), and this elegant biosynthetic story still contains a fly in the ointment, rarely noted. The usual biosynthetic precursor of mevalonate is acetate and so, if the route is correct, acetate should also be a specific precursor of the indole alkaloids. Unfortunately it is not; Leete et al. in 1965 demonstrated the randomization of acetate radioactivity in experiments using *Vinca rosea*, the same plants in which specific incorporations were later demonstrated using other precursors (*3*). To compound the matter further, there is even an example in the literature where acetate fed to *Rauwolfia serpentina* plants gave

[1] Formerly Betty Lem; cf. Ref. (*7*).

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random incorporation into the indole alkaloid ajmaline, but specific incorporation into β-sitosterol (*4*).

Because of these results we have considered the possibility that acetate is not the precursor of mevalonate in indole alkaloid biosynthesis. A search of the literature revealed only leucine as an alternative precursor, mevalonate having been demonstrated to be a metabolic product of leucine (*5*) (see Fig. 1). Furthermore, leucine has been

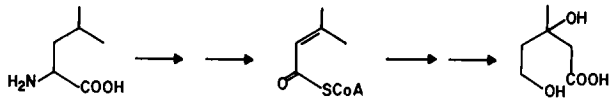
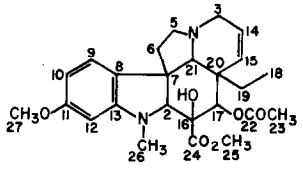
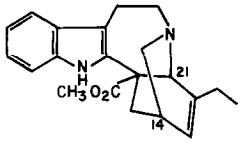


FIG. 1. Conversion of leucine to mevalonic acid via β-methylcrotonyl coenzyme A.



I



II

FIG. 2. Vindoline (**I**) and catharanthine (**II**).

demonstrated to be a precursor of the monoterpene linalool (*6*). Accordingly, we have investigated leucine as a possible precursor of the indole alkaloids vindoline (**I**) and catharanthine (**II**) (see fig. 2) in *Vinca rosea* plants, these alkaloids being examples of Aspidosperma and Iboga alkaloids, respectively.[2]

RESULTS AND DISCUSSION

Initial experiments in this investigation concerned the feeding of ^{14}C-labelled leucine to *Vinca rosea*, with subsequent isolation and radioactive assay of the alkaloids. These results, already published in preliminary form (*7*), showed a small (up to 0.008%) but definite incorporation of radioactivity. Subsequent experiments have raised the level of incorporation to 0.07% for vindoline and 0.02% for catharanthine, levels that are quite comparable with those obtained for acetate feedings in our hands under the same conditions.

From these results it was clear that leucine could be regarded as a possible candidate for the precursor of the nontryptophan portion of the indole alkaloids. The next, and more difficult, step to be tackled was the decision of whether the incorporation is specific or nonspecific and, indeed, whether it is the tryptophan or the nontryptophan portion into which the activity has been transferred. This question of specificity has been approached using both ^{13}C and ^{14}C isotopes.

[2] Ajmalicine is also present in *Vinca rosea* and in some studies has been investigated as an example of a Corynanthe alkaloid. This alkaloid, however, occurs mainly in the roots, and, with the cut shoot feeding method used here, there was insufficient material to obtain data on this alkaloid.

^{14}C Approach

While the advantage of radioactive tracers is sensitivity, the serious disadvantage is that chemical degradation is still the only method to determine the location of the label within a molecule. At the time when this investigation commenced, the only commercially available specifically labelled leucine was *DL*-[2-^{14}C]leucine.[3] If this species were to be converted to mevalonate as in Fig. 1, and thence to the indole alkaloids, activity is expected to be specifically at the 14 and 21 positions of both vindoline and catharanthine. The necessity of chemical degradation is a particular disadvantage in the present case since systematic degradations on neither of these alkaloids has been reported. Of the four possibly labelled carbons in the two alkaloids, only one, C-14 in vindoline, looked to be easily extractable, the other three atoms being at bridgeheads. The obvious first degradative step to remove C-14 of vindoline was oxidation of the double bond. A considerable effort with a variety of oxidants was expended to achieve this oxidation, but, to our surprise, vindoline proved extraordinarily resistant to oxidation, the major product in most reactions being unreacted vindoline; and, in reactions where oxidation was achieved, destruction of the indole ring system also occurred.[4] Even conditions that succeeded for other, closely related, indole alkaloids (e.g., corynantheine) were ineffective in oxidation of vindoline.[5] In view of these results, and the success of the parallel investigation using ^{13}C, this approach was not pursued further.

^{13}C Approach

Lack of sensitivity is the main deterrent to using ^{13}C in biosynthetic studies, especially those with incorporations, like those in the present case, of less than 0.1%. The reward, however, of obtaining the locations(s) of the label directly by ^{13}C nmr spectroscopy are so great that this approach was also pursued. The feeding objective was considered to be the obtainment of 0.5% enrichment of ^{13}C in the alkaloid, corresponding to a 50% signal enhancement in the nmr spectrum. Under these conditions it was felt that the distinction between specific and random incorporation would be unequivocal. Feeding the normal amount of precursor to the plants would not give a result even approaching this level of enrichment. Therefore we considered the question of whether the simple expedient of feeding massive amounts of precursor could overcome this difficulty. Basically this question is twofold, i.e., whether (a) the plant will survive the required dose and (b) whether the percentage incorporation remains constant as the amount of precursor increases, or whether it decreases, thus offsetting any advantage of increased dose. The results summarized in Table 1 using ^{14}C-labelled precursors indicate clearly that in this plant system the percentage incorporation is independent of amount fed, and that the plants can survive this dose size.

[3] The commercial availability of this product now appears to have been discontinued.

[4] Reagents used were OsO_4, $KMnO_4$, $KMnO_4$-crown ethers, Lemieux–von Rudloff reagent, ozone, ruthenium tetroxide. Exhaustive trials with variation of time, temperature, concentration, and work-up procedure were conducted. These experiments and their specific results are summarized in the Ph.D. thesis of Betty Wen (Carleton University, 1977) and are available upon request. We thank Mr. David Dobson for performing some of these oxidations.

[5] We thank Dr. R. T. Brown, University of Manchester, for supplying experimental details of the corynantheine oxidation (cf. Ref. (*8*)).

TABLE 1

PERCENTAGE INCORPORATION AS A FUNCTION OF WEIGHT OF PRECURSOR FED

Weight fed[a] (mg)	Percentage incorporation	
	Vindoline	Catharanthine
10	0.049	0.017
47	0.050	0.024
133	0.052	0.022

[a] Activity fed: 1–6 × 10^8 dpm.

Armed with this result, it is possible to calculate the amount of leucine needed to cause the 0.5% enrichment in the alkaloid required. The actual incorporation based on ^{14}C results is defined as

$$\text{incorporation} = \frac{(\text{total activity})_{\text{alkaloid}}}{(\text{total activity})_{\text{precursor}}}. \quad [1]$$

Translating this into a form appropriate for ^{13}C work, one obtains

$$\text{incorporation} = \frac{\sum (\text{total enrichment at each carbon atom})_{\text{alkaloid}}}{\sum (\text{total enrichment at each carbon atom})_{\text{precursor}}} \quad [2]$$

$$= \frac{\sum (\text{observed enrichment})_{\text{alkaloid}}}{\sum (\text{observed enrichment})_{\text{precursor}}} \times \frac{\text{moles isolated}}{\text{moles fed}}. \quad [3]$$

Defining R_i as the peak size of atom i in the alkaloid, relative to the peak size in the natural abundance spectrum, the percentage of ^{13}C will be ($R_i \times 1.1\%$). The enrichment will thus be $(R_i \times 1.1\%) - 1.1\% = 1.1(R_i - 1)\%$. Thus the $\sum$(observed enrichment)$_{\text{alkaloid}}$ term of Eq. (3) becomes $\sum_i (R_i - 1) \times 1.1\%$.

If the precursor is 90% ^{13}C, the enrichment is $90 - 1.1 = 88.9\%$. Only one carbon of the precursor is labelled. Equation (3) may therefore be modified as follows:

$$\text{incorporation} = \frac{\sum_i (R_i - 1) \times 1.1}{88.9} \times \frac{\text{moles isolated}}{\text{moles fed}}. \quad [4]$$

Multiplying by 100 to obtain the percentage incorporation, one obtains

$$\%\ \text{incorporation} = 1.24 \sum_i (R_i - 1) \cdot \frac{W_{\text{alk}}}{W_{\text{pre}}} \cdot \frac{\text{MW}_{\text{pre}}}{\text{MW}_{\text{alk}}}, \quad [5]$$

where W is the weight of alkaloid and precursor in milligrams. For the purposes of calculating W_{pre}, the weight of precursor required to meet the set requirements, the following values were used: % incorporation (into vindoline) = 0.04%; $R_i = 1.5$ at two carbons (if leucine were to be incorporated specifically), thus $\sum_i (R_i - 1) = 1.0$; $W_{\text{alk}} =$ 40 mg; $\text{MW}_{\text{pre}} = 132$ ($^{12}C_5{}^{13}CH_{13}NO_2$); and $\text{MW}_{\text{alk}} = 456$ (vindoline).

From these values the calculated value of W_{pre} is 359 mg. The values in Table 1 had not reached this level of feeding, but subsequent feedings with ^{14}C-labelled leucine confirmed that the incorporation remained constant even with this amount of precursor fed.

Synthesis of DL-[2-^{13}C]*Leucine*

Having established that the incorporation in the plant and the amount of precursor required were compatible, we took the final preparatory step, involving the selection and synthesis of an appropriate specifically labelled leucine sample. The route in Fig. 3, starting from commercially available acetic acid-[2-^{13}C], was performed. In this

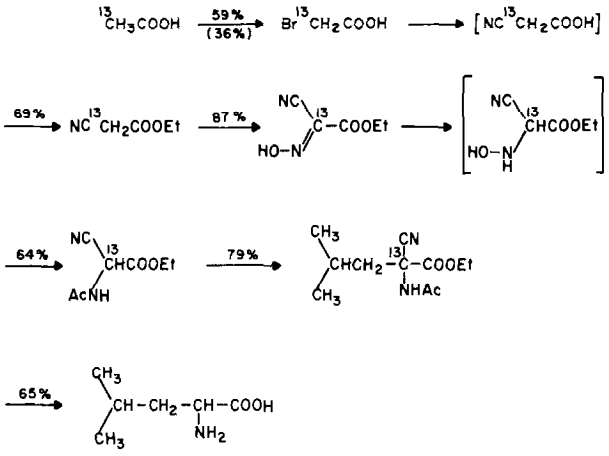


FIG. 3. Synthetic route to DL-[2-^{13}C]leucine.

sequence all the individual steps had either previously been reported in the literature or were adaptations of similar conversions on other compounds (*9–14*). For the isolation of reasonable amounts of leucine the yields of the steps needed to be maintained at or above those previously reported. These yields are shown in Fig. 3; in steps where no yield is shown the product was used directly without isolation, and the yield shown for the next step is an overall yield of the two steps. The yield of the first step (bromination of acetic acid) was significantly increased from 36% (*9*) to 59%; other yields were not significantly different from those previously reported. In the whole sequence 1.96 g of [2-^{13}C]acetic acid was converted to 487 mg of [2-^{13}C]leucine via this eight-stage sequence, an overall yield of 11.5%.

Results of Feeding DL-2-[^{13}C]*Leucine to Vinca rosea*

The [2-^{13}C]leucine, synthesized as described above, was mixed with [1-^{14}C]leucine (1.0×10^8 dpm) before feeding to the plants. The purpose of the small amount of radioactive leucine was to allow normal calculation of incorporation to ensure that in this particular experiment biosynthetic conversion had occurred. The incorporation into vindoline was 0.037%, close to the 0.04% expected; incorporation into catharanthine was too low (<0.01%) to obtain meaningful nmr spectral data, and the investigation was thus restricted to incorporation into vindoline. ^{13}C nmr spectra of natural

abundance vindoline and vindoline from the feeding experiment were measured under the same conditions,[6] and the peak intensities were measured and treated by standard normalization procedures (*16*).

The results obtained, summarized in Table 2, were straightforward and unexpected. Two carbon atoms, C-13 and C-24, were strikingly enriched to the extent of 16 and

TABLE 2

RELATIVE ^{13}C nmr PEAK INTENSITIES OF BIOSYNTHESIZED AND NATURAL ABUNDANCE VINDOLINE

Carbon	Chemical shift (δ_c from TMS)	Relative intensity[a] (%)
2	83.5	—
3	51.2	—
5	52.1	—
6	44.2	—
7	52.9	+10
8	125.2	—
9	122.7	+11
10	104.9	—
11	161.4	+10
12	96.0	—
13	153.9	+16
14	124.0	—
15	130.7	—
16	79.7	—
17	76.5	—
18	7.8	—
19	30.9	—
20	43.1	+6
21	67.3	—
22	171.9	—
23	21.0	+7
24	170.5	+25
25	52.1	—
26	38.2	—
27	55.1	—

[a] Reproducibility, 5%. Peaks for which no increments are given had intensities within experimental error (±5%) of those of natural abundance samples.

25%, respectively; and five other carbon atoms, C-7, C-9, C-11, C-20, and C-23, also appeared to be enriched, but to a lesser extent (6–11%). Equation (5) may be used to calculate the percentage incorporation from nmr data, and the sum of these enhancements gives a value of $\sum(R_i - 1)$ of 0.85, corresponding to an incorporation of 0.05%. This value is in reasonable agreement with the incorporation calculated from ^{14}C data of 0.04%.

[6] Carbon-13 nmr signal assignments for vindoline have previously been made by Wenkert *et al.* (15).

These results are clearly not consistent with the leucine → mevalonate → alkaloid pathway. Of the two most strongly labelled atoms one is in the tryptophan portion and the other, although in the monoterpenoid portion, is not one of the two atoms expected to be labelled. Of the other five atoms with apparent enrichment, only one other (C-20) is in the monoterpenoid portion and again this does not correspond to an expected position. Considering (a) that there is no known direct metabolic process connecting leucine with tryptophan, and (b) that the monoterpene portion is known to arise from two C_5 fragments, this is a result that does not appear to represent any specific biosynthetic route. These results indicate that although isotopic activity is transmitted from leucine to vindoline in *Vinca rosea*, the specific route suggested of simple degradation to mevalonic acid is not a significant pathway followed. Thus although mevalonate is known to be the precursor of the monoterpenoid portion of the indole alkaloids, the awkward position is reached that neither of the two known precursors of mevalonate, acetate and leucine, give experimental results consistent with their so acting in this biosynthetic route.

EXPERIMENTAL

Radioactivity assay. Radioactivity was measured on a Beckman LS-150 liquid scintillation counter, the counting efficiency being determined by external standardization. Low background glass Spectravials (Amersham–Searle) and Spectrafluor (Amersham–Searle) scintillator solution in toluene were used. For alkaloid assay, the alkaloid was dissolved in benzene (1 ml) in a counting vial and the above scintillator solution (15 ml) was added by means of an automatic tilting dispenser. In the case of leucine assays solubilization was achieved using Bio-Solv solubilizer BBS-3 (Beckman) (5%). Counting was performed to 1% statistical accuracy at least three times or until the count remained constant.

Feeding procedure and alkaloid isolation. Shoots of *Vinca rosea* were taken from the plants by cutting diagonally across the basal part of the stems, and the cut end was placed immediately in water. To ensure that airlocks did not develop in the severed end, a second cut was made under the water and the shoots were immediately placed in the feeding solution in a large (25 × 115 mm) test tube. After the plants (70 g) had taken up the solution until there was just enough solution to keep the cut end moist, additional distilled water was added to give about 1 in. of solution. This procedure was repeated several times to ensure that essentially all the feeding precursor had been taken up by the plants. The plants were placed under intermittent fluorescent lamp illumination for the feeding period (2 days to 2 weeks). The shoots were then mascerated in methanol in a Waring or Osterizer blender, suction-filtered, remascerated, and refiltered. The residue was washed with methanol until the filtrate was colourless and discarded. The combined green filtrate (~1.5 liter) was rotary evaporated, and the residue was taken up in 2 *N* hydrochloric acid (100 ml) and filtered. An additional portion of hydrochloric acid (2 *N*, 25 ml) was used to rinse the flask and wash the residue. This aqueous solution was washed with benzene (2 × 70 ml), made basic with 15 *N* ammonium hydroxide, and extracted with chloroform (5 × 50 ml). The combined chloroform extracts were washed with water (75 ml), dried over anhydrous sodium sulfate, and evaporated to give a brown oil (206.3 mg). Radioactivity of the alkaloid mixture was monitored after methanol extraction and after chloroform extraction.

This crude alkaloid mixture was partially purified by dissolving it in chloroform and running it through a short alumina column (5 cm in height) with chloroform (150 ml) to remove the very polar components. The chloroform solution was concentrated again on a rotary evaporator. The yellow residue was dissolved in the minimum amount of benzene and chromatographed on alumina (15 g). The column was eluted successively with petroleum ether 30°/60°, petroleum ether/benzene mixture, benzene, benzene/chloroform mixture, chloroform, and methanol; 35-ml fractions were taken. Every second fraction was checked to tlc (silica/ethyl acetate) with the authentic samples of catharanthine and vindoline as the reference. The later fractions of petroleum ether/benzene (1/1) were combined and crystallized from methanol affording catharanthine (22.09 mg), the middle fractions of benzene/chloroform (9/1) were combined and crystallized from ether giving vindoline (26.99 mg). Recrystallizations and derivativization as the hydrochloride salts were used as purification steps for the alkaloids. The hydrochloride salt of catharanthine was formed by blowing hydrogen chloride gas on the surface of a methanol solution of the alkaloid and was recrystallized from methanol. The hydrochloride salt of vindoline was also made by blowing hydrogen chloride gas on the surface of an ethereal solution of the alkaloid and was recrystallized from acetone.

DL-[2-^{13}C]*Leucine.* [2-^{13}C]Acetic acid (Merck, Sharp and Dohme) was converted to [2-^{13}C]bromoacetic acid using the procedure of Vogel (*9*). The literature yield of 36% (*9*) was improved to 59% principally by using freshly distilled acetic anhydride and pyridine. [2-^{13}C]Bromoacetic acid was converted to [2-^{13}C]cyanoacetic acid and thence directly to ethyl [2-^{13}C]cyanoacetate in 69% overall yield using the procedure of Inglis (*10*). Nitrosation using the conditions of Snyder and Smith (*11*) of nitrosation of diethyl malonate produced ethyl [2-^{13}C]isonitrosocyanoacetate in 87% yield. Conversion of this material to ethyl acetamidocyanoacetate-[2-^{13}C] was achieved in 64% yield using the procedure of Tullar (*12*). Alkylation with isobutyl bromide using the conditions of Albertson and Archer (*13*) gave ethyl [2-^{13}C]2-acetamino-2-cyano-4-methylpentanoate in 79% yield. Hydrolysis of this material with hydrogen bromide according to the procedure of Albertson and Tullar (*14*) gave a 65% yield of [2-^{13}C]leucine, mp 281–283°C sub. (lit. mp 278–283°C (*13*)), no mixed melting point depression; pmr δ_{D_2O}, 1.27 (6*H*, *d*, J = 8 Hz, CH_3's), 1.98 (3*H*, *m*, *CH*–CH_2–), and 4.01 (1*H*, *t*, J = 8 Hz, CH_2–*CH*–COOH). ^{13}C nmr showed one single peak at 53.5 ppm.

Administration of DL-[2-^{13}C]*Leucine to Vinca rosea.* DL-[2-^{13}C]Leucine (375 mg) and DL-[2-^{14}C]leucine (1.01×10^8 dpm) in aqueous solution were fed to *Vinca rosea* plants (145 g) for two weeks. The plants were extracted and the alkaloids were isolated as described above, to give catharanthine (39.3 mg, 8.8×10^3 dpm, 0.009% incorporation) and vindoline (64.5 mg, 3.7×10^4 dpm, 0.037% incorporation). The ^{13}C nmr spectra of this sample of vindoline and of natural abundance vindoline were measured under identical conditions on a Varian XL100 instrument in deuteriochloroform solvent, multiple spectra of each being taken to ensure reproducibility.

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