The Biosynthesis of Monoterpenoid Indole Alkaloids from Strictosidine [1]

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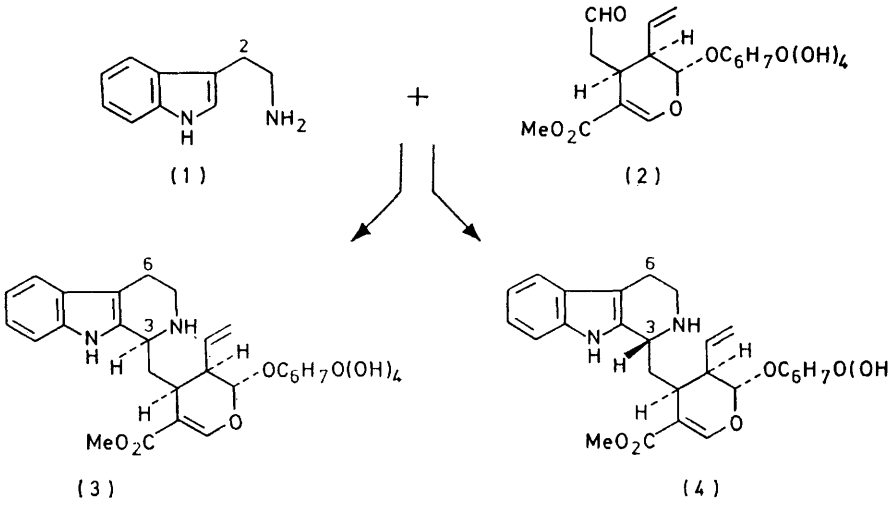
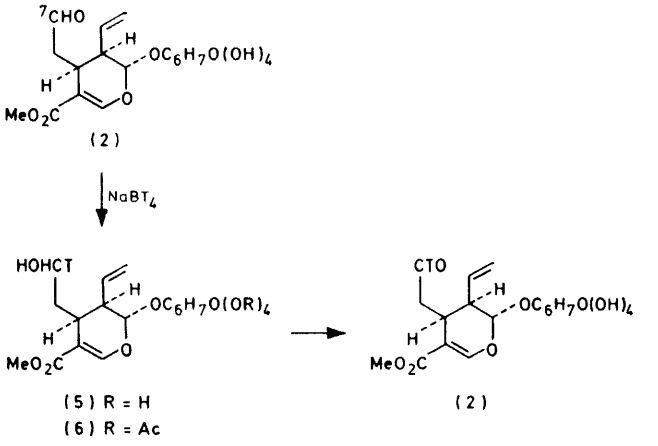
The differential incorporation of doubly labelled strictosidine and vincoside into several indole alkaloids belonging to the *Corynanthe* (3α and 3β series), *Aspidosperma*, and *Iboga* types in three plant families has been studied, and it has been demonstrated that only strictosidine is incorporated while vincoside is metabolically inert in these plants with regard to alkaloid formation. During the conversion of strictosidine into the 3β-indole alkaloids, the hydrogen atom at the 3-position is lost, while it is retained during the biosynthesis of the 3α alkaloids. The chemical synthesis of [7-^{3}H]secologanin, an important intermediate for further work in the biosynthesis of monoterpenoid alkaloids, is also described.

THE fundamental research of Battersby and Arigoni and their associates [2] during the past decade has established the central role of loganin and secologanin (2) in the biosynthesis of a multitude of monoterpenoid indole alkaloids. Secologanin (2) is the ultimate precursor for the C-9/C-10 non-tryptamine carbon skeleton common to the majority of indole alkaloids. Tryptamine (1) and secologanin (2) condense to form an intermediate



which was shown to be biologically rearranged to the diverse structures of the indole alkaloids found in nature.[2] Chemical condensation of (1) and (2) yields a mixture of the two epimers strictosidine (3) and vincoside (4), of which vincoside was shown to be the sole precursor for the biosynthesis of the indole alkaloids.[3] On the other hand, prior to this investigation [3] the other epimer had been isolated from *Rhazia stricta* in high yield, named strictosidine (3), and assumed to occupy a crucial position in the postulated biosynthesis of these alkaloids.[4] The correct stereochemical relationship of the precursor to the products has since then been a matter of controversy and confusion.[5] The absolute stereochemistry of (4) was established unequivocally [6,7] to be (*R*)-3β. This has recently been confirmed by *X*-ray diffraction.[8] Based on this unequivocally established configuration of (4) we were able to prove that, in contrast to previous assumptions,[5] strictosidine (3), with (*S*)-3α stereochemistry, is indeed the key intermediate in the formation of the three classes of monoterpenoid indole alkaloids (*Aspidosperma*, *Iboga*, and *Corynanthe*) in *Catharanthus roseus* (syn. *Vinca rosea*) plants as well as a variety of other species in cell cultures by *in vivo* and *in vitro* techniques.[9,10] The crucial enzyme catalysing the condensation of (1) with (2) to yield exclusively the α-epimer strictosidine (3) has been discovered and named strictosidine synthase.[10] The role of (3) as a precursor *in vivo* has been independently confirmed using *Rhazia* species [11] and *C. roseus*.[12] The above observations were later confirmed [13] also by *in vitro* experiments using *C. roseus* callus tissue. All these results are in accord with reports on the biosynthesis of camptothecin, an alkaloid of taxonomically distant origin, for which strictosidine lactam was previously [14] found to be a precursor and recently [15] also (3). It can therefore be taken for granted, that the α-epimer (3) is the biological precursor for the majority of indole alkaloids, especially those with the 3α-configuration. However, the question remains whether (3) is the universal precursor for all those indole alkaloids which are derived from the condensation of (1) and (2). This latter point is especially important for an alkaloid family with C-3β stereochemistry. Since it has been postulated, based on biomimetic experiments [16] which were assumed to

duplicate the *in vivo* process, that (3) is the precursor for 3α- and (4) for 3β-alkaloids, it has been proposed that vincoside (4) would have a role as an *in vivo* precursor for alkaloids with the C-3β configuration in those plants where these products occur.[12] To test this latter possibility and to gain clarity as to the assumed universal role of (3) as precursor for monoterpenoid indole alkaloids, [^{14}C]-labelled (3) and (4) were fed to a range of plants and their respective incorporation determined. To trace also the fate of 3-H of (3) and (4), a tritium label was introduced into this position. This was achieved by synthesizing [7-^{3}H]secologanin (2), a substance which is also important for future work on the



SCHEME Synthesis of [7-^{3}H]secologenin

biosynthesis of monoterpenoid alkaloids. This labelled secologanin (2) was prepared in satisfactory radiochemical yield by use of sodium borotritiide in the reduction of (2) to the [7-^{3}H]alcohol, named secologanol (5), which was in turn reoxidized to [7-^{3}H]secologanin (2) by treatment with Jones reagent. Previously it has been reported [17] that reduction of secologanin (2) with $NaBH_4$ leads to sweroside. If, however, in this reaction a large excess of borohydride is avoided and the pH is carefully controlled at 7.0 or the reaction is carried out in methanol at 0°, the reaction leads to secologanol (5) as outlined in the Scheme. Condensation of [7-^{3}H]-(2) with tryptamine under standard conditions [3] led to the desired tetrahydro-β-carbolines (3) and (4) labelled at the C-3 position with tritium or the [6-^{14}C]-epimers if [2-^{14}C]tryptamine (1) was used instead. The epimers were separated by chromatography. The synthesis of doubly labelled (3) was achieved by enzymatic condensation of [2-^{14}C]-(1) and [7-^{3}H]-(2) using partly purified strictosidine synthase. In this case the ^{3}H and ^{14}C labels were within the same molecule. The position of the tritium label in compound (3) has been proven previously [1] to be in the desired 3-position. The stereochemical assignment of the products (3) and (4) to the α- or β-series was achieved by several methods.[10] The c.d. spectra of the acetylated products and the n.m.r. spectra of the lactams derived from these products [10,18] showed clearly their stereochemical relationship; *e.g.* only the α-epimer gives the anomalous acetate signal at δ 1.23.[6,19,10] Furthermore it was found [10] that t.l.c. of the β-epimer with a solvent system containing diethylamine converts it quantitatively into the lactam, while the α-epimer under these conditions is stable and easily separable from the β-lactam. This marked difference in the stability of the α- and β-epimers is in full agreement with previous observations.[3]

In a preliminary experiment [7-^{3}H]secologanin (2) and [2-^{14}C]tryptamine (1) (as an internal check) were fed simultaneously to *Rauwolfia canescens* which contains both 3α- and 3β-alkaloids. Under the conditions chosen, no chemical condensation of both compounds occurred in the feeding solution. As can be seen in Table 1, [^{14}C]tryptamine (1) was incorporated into all four alkaloids (7)—(10) examined; interestingly enough, however, all the 3α-alkaloids contained a relatively high ^{3}H : ^{14}C ratio, while the 3β-alkaloid (10) showed a very low isotope ratio suggesting that the tritium label was lost to a large extent. Thus, already at this stage there was an indication that the 3β-reserpiline (10) is not formed from (4) since in this case the ^{3}H label should be retained to a similar extent as in the 3α-series. The observed considerable variation in the ^{3}H : ^{14}C ratio of the 3α-alkaloids may reflect a differential uptake and internal dilution rate for both precursors.

The decisive test was carried out by using doubly labelled [3-^{3}H,6-^{14}C]strictosidine (3) which was fed to *R. canescens* shoots to afford the incorporation recorded in Table 2. The ^{14}C label of (3) was incorporated satisfactorily into all five alkaloids under investigation. However, only the 3α-alkaloids showed the same ^{3}H : ^{14}C ratio as the precursor, while both alkaloids (10) and (11) with the β-configuration lost the tritium label to a large extent. The same phenomenon was found if the double labelled strictosidine (3) is supplied to *Mitragyna speciosa* which contains, among other alkaloids, mitragynine (13) and speciociliatine (14) which differ only in the stereochemistry of 3-H. In this plant also, as for

TABLE 1

Simultaneous application of [7-^{3}H]secologanin and [2-^{14}C]tryptamine to young shoots of *R. canescens*

Total activity fed ^{3}H-Secologanin 44.10 × 10^{6} d.p.m. (69.38 × 10^{6} d.p.m. μmol^{-1})
^{14}C-Tryptamine 10.48 × 10^{6} d.p.m. (13.88 × 10^{6} d.p.m. μmol^{-1})

Alkaloid isolated	^{3}H Stereochemistry.	^{14}C Incorporation (%)	^{14}C Specific activity (d.p.m. μmol^{-1})	^{3}H : ^{14}C
α-Yohimbine (7)	α	0.14	1.18 × 10^{4}	8.15 : 1
Isoreserpiline (8)	α	0.05	3.47 × 10^{3}	14.82 : 1
Aricine (9)	α	0.02	1.24 × 10^{3}	10.49 : 1
Reserpiline (10)	β	0.04	2.91 × 10^{3}	0.98 : 1

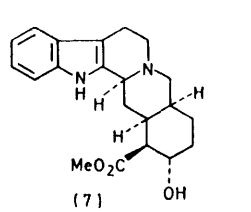
TABLE 2

[3-^{3}H,6-^{14}C]Strictosidine feeding experiments in different *Apocynaceae* plants

Plant	Alkaloid isolated	^{3}H Stereochemistry	% (^{14}C)	^{14}C Specific activity (d.p.m. μmol^{-1})	Precursor ^{3}H : ^{14}C	Product ^{3}H : ^{14}C
R. canescens [a]	α-Yohimbine (7)	α	1.63	6.94×10^4	5.45 : 1	5.82 : 1
R. canescens [a]	Isoreserpiline (8)	α	1.46	3.14×10^4	5.45 : 1	5.67 : 1
R. canescens [a]	Aricine (9)	α	0.79	1.43×10^4	5.45 : 1	5.57 : 1
R. canescens [a]	Reserpiline (10)	β	0.56	2.28×10^4	5.45 : 1	0.10 : 1
R. canescens [a]	Isoreserpinine (11)	β	0.16	5.28×10^4	5.45 : 1	0.43 : 1
M. speciosa [b]	Mitragynine (13)	α	2.72	3.20×10^5	7.17 : 1	8.40 : 1
M. speciosa [b]	Speciociliatine (14)	β	0.53	5.74×10^4	7.17 : 1	0.10 : 1
R. vomitoria [a]	Ajmaline (15)	α	0.72	2.11×10^3	5.45 : 1	4.99 : 1
C. roseus [b]	Ajmalicine (12)	α	0.46	2.03×10^4	7.17 : 1	7.07 : 1
C. roseus [b]	Catharanthine (16)		0.03	n.d.[c]	7.17 : 1	7.35 : 1
C. roseus [b]	Vindoline (17)		0.19	n.d.[c]	7.17 : 1	7.36 : 1

[a] Total activity fed: 12.10×10^6 d.p.m. ^{3}H; 2.22×10^6 d.p.m. ^{14}C. [b] Total activity fed: 15.91×10^6 d.p.m. ^{3}H; 2.22×10^6 d.p.m. ^{14}C. [c] Not detected.

the *Rauwolfia* alkaloids, during the biosynthesis of the α-epimer the ^{3}H label was retained while the β-alkaloid lost the ^{3}H atom (Table 2). This clearly demonstrates that 3α- as well as 3β-alkaloids are derived from strictosidine (3) and that the biosynthetic conversion of (3) into the 3β-alkaloids proceeds with loss of hydrogen at C-3, while it is retained in the formation of the 3α-series. Further. to demonstrate that strictosidine (3) is a general precursor this compound was fed to *Rauwolfia vomitoria* and *C. roseus*. The results of this double-labelling experiment showed that the main skeleton of (3) is incorporated intact without appreciable change in the ^{3}H : ^{14}C ratio (Table 2). The incorporation of the C-3 tritium atom of (3) in the *C. roseus* alkaloids (12), (16), and (17) is in agreement with the earlier observation that [7-^{3}H]loganin was incorporated [which labels the formyl hydrogen in secologanin and thus 3-H of (3)] into these alkaloids and that no hydrogen migration was involved during the biosynthesis of these compounds.[20] Furthermore [6-^{14}C]strictosidine (3) was transformed into gelsemine (18) in *Gelsemium sempervirens* (0.47% incorporation), into strychnine (19) (0.12% incorporation) in *Strychnos nux-vomica*, and into vincadifformine (20) (0.14% incorporation) in *Vinca major*. In addition, feeding of [6-^{14}C]-(3) resulted in the formation of heavily labelled alkaloids of *Rhazia stricta*, *Rhazia orientalis*, *Amsonia tabernaemontana*, *Vallesia glabra*, *Cinchona pubescens*, and *Uncaria gambir*; the identification of the labelled compounds is in progress. In all these cases parallel feeding experiments were performed using [3-^{3}H,6-^{14}C]vincoside (4) of similar specific activity as that of (3) used in Table 2. However, in none of the investigated plants was any incorporation of vincoside (4) into the alkaloid fractions (detection limit <0.001% incorporation) observed. Therefore it can be stated that within the four plant families thus far investigated *Apocynaceae*, *Loganiaceae*, *Rubiaceae*, and *Nyssaceae*,[15] strictosidine (3) is the sole biosynthetic precursor for the elaboration of the monoterpenoid indole alkaloids derived from the condensation of tryptamine (1) with secologanin (2). Furthermore strictosidine (3) gives rise not only to *Corynanthe* (3α- and 3β-series), *Iboga*, and *Aspidosperma* type alkaloids but also to more complex structures such as those of strychnine, gelsemine, and probably quinine. Strictosidine (3) can therefore be regarded as the universal precursor for monoterpenoid indole alkaloids.



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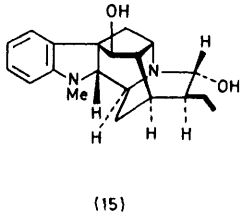

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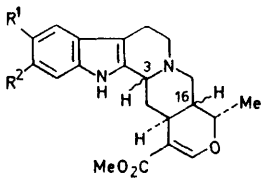
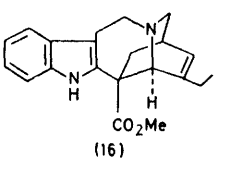


(8) $R^1 = R^2 = OMe, 3\alpha, 16\alpha$
(9) $R^1 = OMe, R^2 = H, 3\alpha, 16\alpha$
(10) $R^1 = R^2 = OMe, 3\beta, 16\alpha$
(11) $R^1 = H, R^2 = OMe, 3\beta, 16\alpha$
(12) $R^1 = R^2 = H, 3\alpha, 16\beta$



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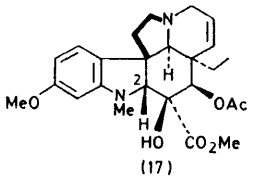

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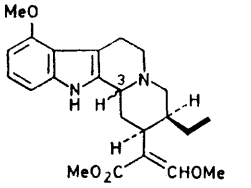
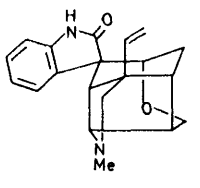


(13) 3α
(14) 3β



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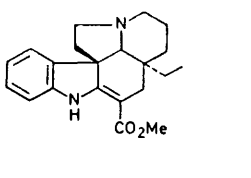

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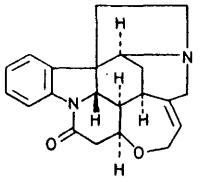

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EXPERIMENTAL

General Directions.—Unless otherwise stated u.v. spectra (Beckmann model 24) and optical rotations (Perkin-Elmer

model 141) were determined for solutions in methanol. I.r. spectra were taken for KBr discs and n.m.r. spectra for solutions in deuteriochloroform. T.l.c. was carried out on silica SIL G/UV_{254} (Machery-Nagel) Polygram plates, if not otherwise stated. Proportions given for mixed solvents are by volume.

Measurement of Radioactivity.—Liquid scintillation counting was used for the measurement of ^3H and ^{14}C activities (Berthold liquiszint BF 5000). Radioactive samples were dissolved in methanol and liquid scintillator added (10 ml); quoted activities were corrected by the internal standard method for self absorption. The relative efficiencies were obtained by counting [*U*-^3H]- and [*methyl*-^{14}C]-toluene standards (Amersham). T.l.c. plates were scanned with a Berthold DC II instrument.

Plant Material.—The experimental plants were grown in a greenhouse, in most cases from seeds. The plants were later transferred to a Phytotron chamber at 28 °C at 75% relative humidity and 10 000 lux light in a 16 h day, 8 h night photoperiod. Single apical cuttings *ca.* 5—10 cm in length of vigorously growing plants were used for the feeding experiments.

Feeding Techniques.—In the case of *Strychnos* and *R. vomitoria* growing seedlings with intact root systems were fed by the capillary feeding technique,[21] In all other cases the labelled compounds (1.16 μmol) were fed through the freshly cut ends of shoots. The labelled compounds were dissolved in water (0.5 ml) containing up to 5% ethanol. When uptake was complete, water was added periodically. The plants were allowed to metabolize for 24 h under the climatic conditions stated above and were then analysed for alkaloids.

Isolation and Purification of Alkaloids.—The plant material (typically 0.5 g fresh weight) was cut into small pieces and exhaustively extracted with boiling 80% aqueous ethanol. The extract was taken to dryness, the residue taken up in methanol, and an aliquot portion subjected to t.l.c. Zones containing alkaloid were eluted and rechromatographed in the subsequent solvent system. The systems for the *Rauwolfia* alkaloids were: acetone–light petroleum (b.p. 40—60°)–diethylamine 20 : 70 : 10; xylene–diethylamine 8 : 2; and cyclohexane–chloroform–diethylamine 5 : 4 : 1. For the *Catharanthus* alkaloids: ethyl acetate–ether–light petroleum (b.p. 40—60°) 20 : 20 : 40; chloroform–n-hexane–acetone 25 : 38 : 20; and ethyl acetate–ether–n-hexane 20 : 20 : 8; for *Mitragyne* alkaloids: ethyl acetate–propan-2-ol–NH_3–n-hexane 56 : 1 : 0.5 : 36; xylene–methyl ethyl ketone–diethylamine 75 : 35 : 2; ether–diethylamine 95 : 5; chloroform–diethylamine 9 : 1.

Purification to constant specific activity was followed by u.v. spectroscopy. If necessary the alkaloids were diluted with carrier material and crystallized to constant specific activity following published procedures.[22]

Preparation and Synthesis of Compounds.—Secologanin was isolated from *Lonicera tartarica* by the procedure of Hutchinson as given in ref. 23.

Secologanol (5). Sodium borohydride (80 mg) was added in small portions to a solution of secologanin (500 mg) in methanol (5 ml) at 0° under stirring. After 10 min the mixture was slightly acidified with acetic acid. The solution was evaporated to near dryness and the residue chromatographed on preparative plates (GF_{254}; 1 mm; solvent acetone–chloroform–water 80 : 20 : 5). The band of R_F 0.30 was eluted and rechromatographed (Polygram) in the same solvent system (R_F 0.35). Pure secologanol was obtained as a *powder* (300 mg) (Found: C, 50.4; H, 6.8. $C_{17}H_{26}O_{10},H_2O$ requires C, 50.0; H, 6.9%); $\nu_{max.}$ 3 330, 1 685, and 1 627 cm^{-1}; $[\alpha]_D^{25}$ −127° (*c* 0.95); $\lambda_{max.}$ 237 nm (log ε 4.01).

Penta-acetylsecologanol (6). A solution of secologanol (35 mg) in pyridine (1 ml) and acetic anhydride (1 ml) was allowed to react overnight at room temperature. The solution was then added to ice–water and evaporated. The residue was chromatographed (t.l.c. solvent chloroform–methanol 98 : 2). The main band was eluted and recrystallized from methanol (37 mg), m.p. 130—131° (Found: C, 54.4; H, 6.0. $C_{27}H_{36}O_{15}$ requires C, 54.0; H, 6.0%); $\nu_{max.}$ 1 733, 1 700, and 1 628 cm^{-1}; $[\alpha]_D^{25}$ −91.7° (*c* 0.67 in chloroform); $\lambda_{max.}$ 232 nm (log ε 4.03); *m/e* 600 (M^+, 0.03%), 540 (1), 331 (14), 192 (32), 169 (100), and 115 (14), the molecular ion was determined using the internal standard method; δ 1.93 (3 H, s), 1.99 (3 H, s), 2.01 (3 H, s), 2.04 (3 H, s), 2.09 (3 H, s), 3.70 (3 H, s), and 7.36 (1 H, d, *J* 0.8 Hz).

Synthesis of Precursors.—[7-^3H]*Secologanol* (5).—A solution of secologanin (39 mg) in methanol (0.5 ml) was added to sodium borotritiide (100 mCi; 0.34 mg) at 0°. After 10 min at 0° [7-^3H]secologanol was worked-up and purified by t.l.c. as above, radiochemical yield 59.0 mCi.

[7-^3H]*Secologanin* (2). [7-^3H]Secologanol (7.8 mg; 2 mCi) was dissolved in a mixture of methanol (0.2 ml) and acetone (0.7 ml) at 0°. To this mixture Jones reagent [24] (0.4 ml) diluted 1 : 6 with cold acetone was added slowly with stirring at 0°. After 30 min and again after 60 min, diluted Jones reagent (0.2 ml each time) was added. After a further 30 min the solvent was removed under nitrogen and the mixture subjected to t.l.c. (solvent acetone–chloroform–water 80 : 20 : 5). The band containing secologanin (R_F 0.35) was eluted and subsequently rechromatographed (solvent chloroform–n-propanol–methanol–water 45 : 15 : 60 : 40; chloroform phase, R_F 0.66). The yield was 0.41 mCi (41%). Unchanged [7-^3H]secologanol was recovered (1 mCi) after the first t.l.c. The position of the label in secologanin was determined by the formation of secologanin tetra-acetate and further oxidation to secoxyloganin tetra-acetate [25] upon which all [^3H] label was lost.

[6-^{14}C]*Strictosidine* (3) *and* [6-^{14}C]*vincoside* (4). To a solution of [2-^{14}C]tryptamine bisuccinate (50 μCi; 1.44 mg) in 1M-citrate buffer (pH 4.0; 0.1 ml) secologanin (48 mg) was added. The mixture was allowed to stand for 48 h at room temperature in the dark. [6-^{14}C]-(3) and -(4) were isolated by t.l.c. Plates were developed twice using ether–ethanol 4 : 1 as solvent, which separated both epimers [(3) R_F 0.34; (4) R_F 0.26]. The yield of each epimer was 15%.

The same procedure was followed for the preparation of a mixture of [3-^3H]strictosidine (3) and [3-^3H]vincoside (4); however, the mole ratio of tryptamine to [7-^3H]secologanin was changed to 12 : 10.

[3-^3H,6-^{14}C]*Strictosidine* (3). In a typical experiment [2-^{14}C]tryptamine (50 μCi; 7 μmol) and [7-^3H]secologanin (273 μCi, 7 μmol) were dissolved in a total volume of 5 ml of 0.5M-potassium phosphate buffer (pH 7.0). Partially purified strictosidine synthase (4.34 mg; 2 670 pkat mg^{-1}) was added and the mixture incubated at 30° for 2 h. The mixture was subsequently freeze-dried and the residue extracted with ethanol (3 × 0.5 ml). The organic phase was chromatographed (solvent acetone–methanol–diethyl-

amine 70 : 10 : 10). The band containing strictosidine (R_F 0.50) was eluted with methanol and rechromatographed (solvent chloroform–methanol 4 : 1), R_F 0.25. The yield of doubly labelled product was 80.5 μCi ^3H and 14.77 μCi ^{14}C (overall yield in several runs 30%); specific activity 39.0 μCi μmol^{-1} ^3H and 7.14 μCi μmol^{-1} ^{14}C. The specific activity was lowered if necessary by addition of unlabelled strictosidine.

Degradation of Doubly Labelled Ajmalicine.[26]—Ajmalicine (12) (27 526 d.p.m. μmol^{-1} ^3H; 3 894 d.p.m. μmol^{-1} ^{14}C; ^3H : ^{14}C 7.07 : 1) which was obtained from the feeding experiments of *C. roseus* using [3-^3H,6-^{14}C]-(3) as precursor (Table 2) was dissolved in a mixture of mercury(II) acetate (4 mg) in acetic acid (0.2 ml). The mixture was heated for 2 h at 60°. The solution was taken to near dryness and subjected to t.l.c. (xylene–methyl ethyl ketone–diethylamine, 70 : 35 : 2). Dehydroajmalicine (R_F 0.76) was clearly separated from ajmalicine (R_F 0.68). Dehydroajmalicine was eluted with acidified methanol and the solvent evaporated to dryness. The residue was dissolved in methanol (0.5 ml), sodium borohydride was then added (5 mg), and after 10 min the product was purified by t.l.c. as above. The recovered ajmalicine (1 345 d.p.m. μmol^{-1} ^3H; 3 282 d.p.m. μmol^{-1} ^{14}C) showed a ^3H : ^{14}C ratio of 0.41 : 1.

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