Pyrrolizidine Alkaloid Biosynthesis. Synthesis of ^{13}C-Labelled Putrescines and their Incorporation into Retronecine[1]

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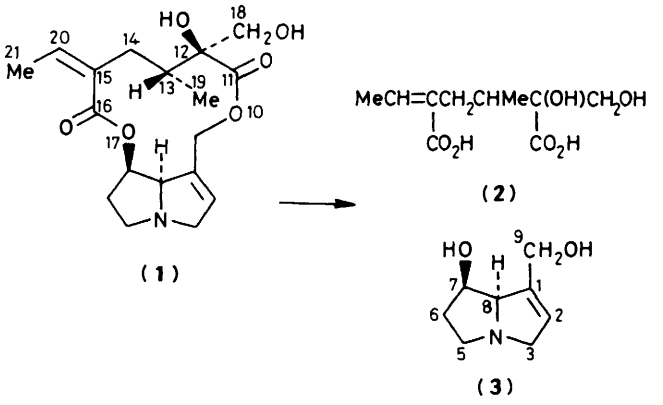
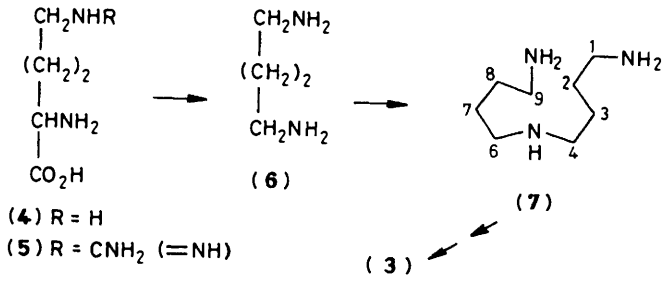
The biosynthesis of the retronecine (**3**) portion of the pyrrolizidine alkaloid retrorsine (**1**) has been studied in *Senecio isatideus* plants using ^{13}C-labelled putrescines. Samples of [1-^{13}C]- (**10**), [1,4-$^{13}C_2$]- (**8**), and [2,3-$^{13}C_2$]-putrescine (**9**) were prepared and used to obtain labelled retronecine. The labelling patterns, determined by ^{13}C n.m.r. spectroscopy, demonstrated that two molecules of putrescine are incorporated to about the same extent into each molecule of retronecine. [1-*amino*-^{15}N,1-^{13}C]Putrescine (**11**) was synthesized and fed to *S. isatideus* plants. The labelling pattern in the retronecine (**12**) produced was consistent with the formation of a later C_4–N–C_4 symmetrical intermediate in retronecine biosynthesis.

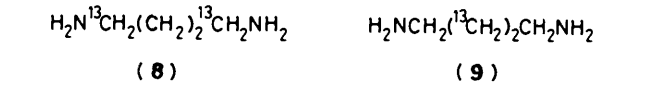
Many pyrrolizidine alkaloids, including retrorsine (**1**), contain retronecine (**3**) as the base (necine) portion.[2] Alkaline hydrolysis of retrorsine, the major alkaloidal constituent of *Senecio isatideus* plants, yields isatinecic acid (**2**) and retronecine (Scheme 1).[3] Experiments with radiolabelled precursors have shown that ornithine (**4**),[4–7] arginine (**5**),[6,7] putrescine (**6**),[5,7] spermidine,[7] and spermine,[7] are specific precursors of retronecine (Scheme 2). Retronecine is also known to be derived from L-ornithine and L-arginine[8] but not from the D-enantiomers. However, incomplete labelling patterns have been obtained for the distribution of the ^{14}C and ^{3}H labels,[7,9] because it has not been possible to degrade retronecine completely to isolate each carbon atom separately. High total incorporations (1.6—5.2%) of ^{14}C-labelled precursors into retrorsine (**1**) were recently obtained by us, using putrescine, spermine, and spermidine.[7] These results suggested that ^{13}C-labelled putrescines should be synthesized and could then be used to produce ^{13}C-labelled retrorsine and retronecine. The labelling patterns in these alkaloids could then be established by ^{13}C n.m.r. spectroscopy.



Scheme 1. *Reagents:* i, $Ba(OH)_2$



Scheme 2.



Results and Discussion

The dihydrochloride of [1,4-$^{13}C_2$]putrescine (**8**) was prepared by treatment of 1,2-dibromoethane with potassium [^{13}C]-cyanide, followed by reduction of the succinonitrile with borane in tetrahydrofuran, and acidification of the product with hydrochloric acid. The best incorporations of this ^{13}C-labelled precursor (**8**) into retrorsine (**1**) were obtained by pulsed feeding of sterile aqueous solutions of the precursor into the xylems of *Senecio isatideus* plants through stem punctures.[7] Each sample of ^{13}C-labelled precursor was 'spiked' by addition of [1,4-^{14}C]putrescine dihydrochloride. One week after completion of the pulsed feeding, the plants were harvested, and retrorsine (**1**) was isolated and recrystallized to constant specific radioactivity and the specific ^{14}C-incorporation† was calculated (Table 1). Alkaline hydrolysis of the samples of retrorsine gave retronecine (**3**) (isolated as its hydrochloride) and isatinecic acid (**2**). These products were recrystallized to constant specific radioactivity. In each experiment, more than 95% of the ^{14}C radioactivity was located in the retronecine hydrochloride (Table 2).

The ^{13}C n.m.r. spectrum of retronecine hydrochloride in deuterium oxide was assigned using off-resonance and single-frequency decoupled spectra. Comparison of the $^{13}C\{^1H\}$ n.m.r. spectra for ^{13}C-labelled retronecine hydrochloride (Table 1, experiments 1—4) with unlabelled material run under the same conditions (Figure 1a), showed enhanced signals for C-5, C-9, C-3, and C-8. However, the signals due to C-5 and C-8 were broadened owing to ^{13}C–N–^{13}C (and, possibly, ^{13}C–C–C–^{13}C) coupling arising from intramolecular coupling in the corresponding C_4 unit derived from a single molecule of ^{13}C-labelled putrescine (**8**). The broadening in the signals due to C-9 and C-3 could also arise from ^{13}C–C–C–^{13}C coupling or it could be geminal coupling (C-3 to C-5 or C-8 of retronecine, and C-9 to C-8) resulting from combination of two molecules of ^{13}C-

† Specific ^{14}C incorporation per C_4 unit is calculated from [(molar activity of product × ½)/(molar activity of precursor)] × 100%.

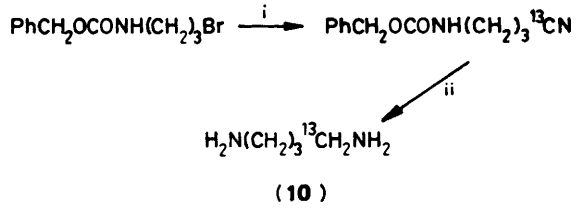
Table 1. Incorporation of precursors into retrorsine (**1**) in *Senecio isatideus* plants

Expt.	Precursor	Quantity fed/ mg	No. of plants	Age of plants/ months	Length of expt./ days	% ^{14}C Specific incorporation[a] in (**1**)
1	(**8**)	80	2	6	21	0.48
2	(**8**)	95	6	4	28	0.3
3	(**8**)	40	4	2	14	1.2
4	(**8**)	60	10	2	10	1.5
5	(**10**)	160	10	2	21	1.9
6	(**10**)	160	10	4	21	0.9
7	(**10**)	160	25	2	20	2.9
8	(**10**)	160	102	1	10	4.5
9	(**9**)	50	4	5	14	0.25
10	(**11**)	25	2	3	14	1.1

[a] See footnote † on p. 101.

Table 2. Distribution of ^{14}C radioactivity in retrorsine (**1**) and incorporation of ^{13}C into retronecine (**3**)

Expt.	% Radioactivity in isatinecic acid (**2**)	% Radioactivity in retronecine (**3**)	Average enrichment factor per labelled site	% ^{13}C Specific incorporation per C_4 unit
1	3	99	0.2 ± 0.1	0.4
2	1	95	0.1 ± 0.1	0.2
3	0	96	0.5 ± 0.1	1.1
4	2	100	0.8 ± 0.1	1.7
5	2	95	0.4 ± 0.1	1.8
6	1	97	0.2 ± 0.1	0.8
7	1	102	0.6 ± 0.1	2.6
8	0	99	1.0 ± 0.1	4.3
9	3	97	0.17 ± 0.02	0.2
10	2	101	0.45 ± 0.05	1.0



(**10**)

Scheme 3. *Reagents:* i, $K^{13}CN$; ii, H_2, Pd/C

labelled putrescine (**8**) in the biosynthetic pathway.* This broadening of the signals made the measurement of the enrichment factors† for the labelled carbon atoms in retronecine difficult.

Accordingly [1-^{13}C]putrescine (**10**) was synthesized (Scheme 3) by treatment of the *N*-benzyloxycarbonyl derivative of 1-amino-3-bromopropane with potassium [^{13}C]cyanide to give the corresponding nitrile. Catalytic hydrogenation then yielded [1-^{13}C]putrescine (**10**) isolated and recrystallized as its dihydrochloride. A number of feeding experiments were carried out as described above using the different conditions summarised in Table 1 (experiments 5—8). The highest ^{14}C specific incorporations‡ were obtained by feeding large numbers of freshly rooted cuttings for a short period with relatively large quantities of precursor. In the $^{13}C\{^1H\}$ n.m.r. spectrum of retrorsine obtained in experiment 8 and run in deuteriochloroform, four enhanced signals were observed for C-5, C-9, C-3, and C-8 at δ 53.0, 61.0, 62.9, and 77.5 p.p.m. This sample was hydrolysed to give retronecine hydrochloride which displayed enhanced ^{13}C signals due to the same four carbon atoms (Figure 1b). The average enrichment factor† for each labelled position was 1.0 ± 0.1% ^{13}C. The total enrichment per C_4 unit is therefore 2.0% ^{13}C and the estimated ^{13}C specific incorporation is (2.0 × 2/93) × 100% = 4.3% per C_4 unit of putrescine (93/2 atoms % ^{13}C is the average enrichment at each labelled position of retronecine). In each of the experiments 1—8 the enhancements of the four signals for C-3, C-5, C-8, and C-9 were nearly equal. Some broadening at the base of these enriched signals was still observed, again probably due to geminal coupling arising from the combination of two ^{13}C-labelled putrescine-derived C_4 units.*

In many of the preceding experiments, ^{13}C enrichments were low, mainly owing to the dilution of the labelled alkaloid with appreciable quantities of endogenous unlabelled retrorsine. Enrichment factors were therefore difficult to estimate. The use of our best conditions with large numbers of young plants is not always feasible. We therefore decided to synthesize a ^{13}C–^{13}C doubly labelled precursor. Feeding experiments could then be carried out with smaller amounts of precursor on well established plants. Low enrichments could be more readily

* The biosynthetic samples are a mixture of ^{13}C-enriched material and material at natural abundance which was present in the plants at the beginning of the feeding experiment. The combination of two molecules of ^{13}C-labelled putrescines is rendered likely by the technique of pulsed feeding in which relatively large quantities of ^{13}C-labelled putrescines are added to the plants for a short period. The resulting dilution by unlabelled endogenous putrescine could be quite small.

† The enrichment factor for each labelled site in retronecine (**3**) is the excess of ^{13}C above natural abundance and is calculated as [(integral of labelled site − natural abundance integral or integral of doublet signals)/(natural abundance integral)] × 1.1%.

‡ See footnote † on p. 101.

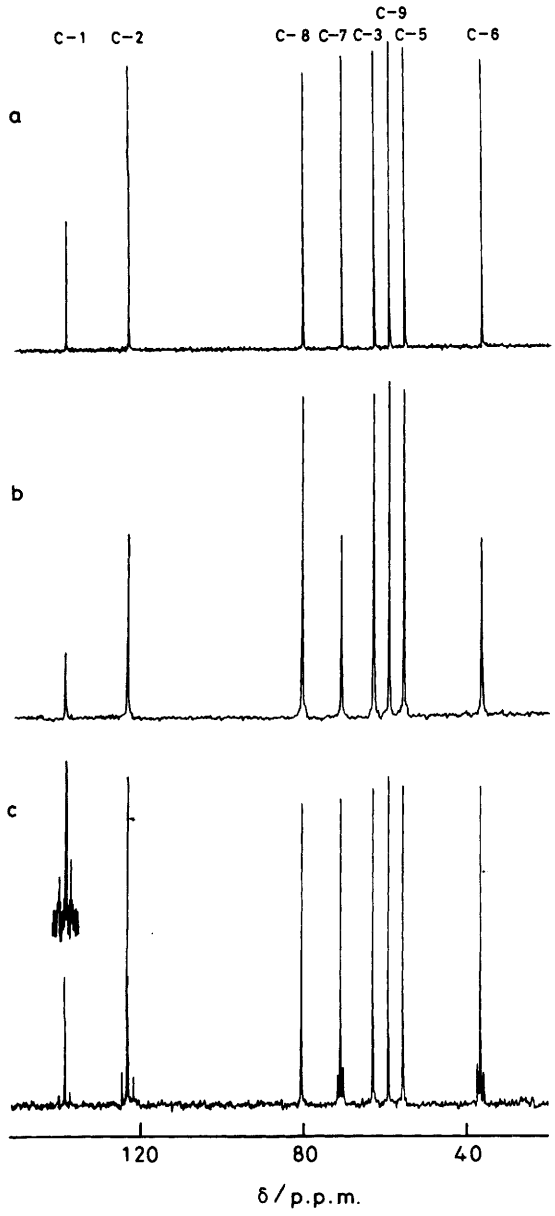
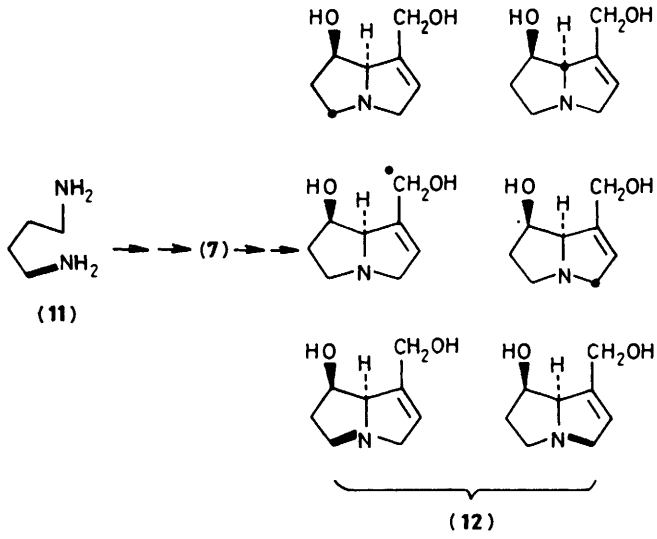


Figure 1. Proton-noise decoupled ^{13}C n.m.r. spectra of retronecine (**3**) hydrochloride in deuterium oxide: (a) unlabelled material; (b) enriched with [1-$^{13}C_1$]putrescine (**10**) dihydrochloride; (c) enriched with [2,3-$^{13}C_2$]putrescine (**9**) dihydrochloride

identified by observation of ^{13}C–^{13}C doublets in the ^{13}C n.m.r. spectrum of the alkaloid produced. The dihydrochloride of [2,3-$^{13}C_2$]putrescine (**9**) was synthesized from [1,2-$^{13}C_2$]-1,2-dibromoethane by treatment with potassium cyanide, followed by reduction of the succinonitrile, and conversion of the labelled putrescine into its dihydrochloride. This material was fed to four strongly growing, mature plants of *Senecio isatideus* (experiment 9). A relatively low ^{14}C specific incorporation* of 0.25% per C_4 unit was obtained in retrorsine. The $^{13}C\{^1H\}$ n.m.r. spectrum of retrorsine in deuteriochloroform showed a pair of doublets at δ 34.7 (*J* 33.8 Hz) and 75.1 p.p.m. (*J* 34.0 Hz), corresponding to C-6 and C-7, and a pair of doublets at δ 131.4 (*J* 70.0 Hz) and 136.7 p.p.m. (*J* 70.2 Hz) corresponding to C-1 and



Scheme 4.

C-2 respectively of retrorsine (**1**). These observations, together with a Distortionless Enhancement by Polarization Transfer (DEPT) experiment on unlabelled retrorsine, permit the first correct assignment of the ^{13}C n.m.r. spectrum of retrorsine (**1**) (see Experimental section). It should be noted that the previous assignments of the ^{13}C n.m.r. spectrum of retrorsine all contain at least one pair of carbons that are wrongly assigned.[10–13] In the earliest assignment of this spectrum,[14] most of the carbon atoms of retrorsine are incorrectly assigned.

The sample of retrorsine (experiment 9) was hydrolysed to yield retronecine hydrochloride. The $^{13}C\{^1H\}$ n.m.r. spectrum of this material (Figure 1c) showed the same two pairs of doublets at δ 137.4 and 122.1 p.p.m. (*J* 71 Hz), and δ 35.9 and 70.1 p.p.m. (*J* 34 Hz) with enrichment factors of 0.16 and 0.18 ± 0.02% ^{13}C respectively. The average enrichment factor is 0.17% ^{13}C, corresponding to an estimated ^{13}C specific incorporation of (0.17 × 1/81) × 100% = 0.21% per C_4 unit [81 atom % ^{13}C is the average enrichment due to $^{13}C_2$ species at each labelled position of putrescine (**9**)].

All four ^{13}C-labelled sites in each sample of ^{13}C-labelled retronecine (experiments 1—9) display nearly equal enrichment factors. This observation supports the theory that two molecules of putrescine combine to form a later C_4–N–C_4 symmetrical intermediate, such as homospermidine (**7**), which is then converted into retronecine (Scheme 2). We believed that the use of [^{13}C-^{15}N]-labelled putrescine would provide further evidence for this later symmetrical intermediate. Biosynthesis of retronecine (**3**) from [^{13}C-^{15}N]-labelled putrescine *via* a symmetrical intermediate such as homospermidine (**7**) should lead to a $^{13}C\{^1H\}$ n.m.r. spectrum of retronecine which contains two ^{13}C–^{15}N doublets associated with C-3 and C-5 (Scheme 4).[15] A sample of [1-*amino*-^{15}N, 1-^{13}C]putrescine (**11**) was prepared as outlined in Scheme 3 using potassium [^{13}C,^{15}N]cyanide to introduce the contiguous labels. The feeding experiment was carried out (experiment 10) and retrorsine was isolated with a ^{14}C specific incorporation* of 1.1% per C_4 unit. Retronecine hydrochloride was obtained by alkaline hydrolysis followed by acidification. The $^{13}C\{^1H\}$ n.m.r. spectrum of this material showed enrichment factors of 0.4 ± 0.05% ^{13}C for the signals due to C-5 and C-8 at δ 54.9 and 79.6 p.p.m. respectively, and 0.5 ± 0.05% ^{13}C for the signals at δ 62.2 and 58.5 p.p.m. due to C-3 and C-9. This corresponds to an average enrichment factor of 0.45% ^{13}C for each labelled site, and a specific ^{13}C incorporation per C_4 unit of (0.45 × 2/90.6) = 0.99% [90.6/2

* See footnote † on p. 101.

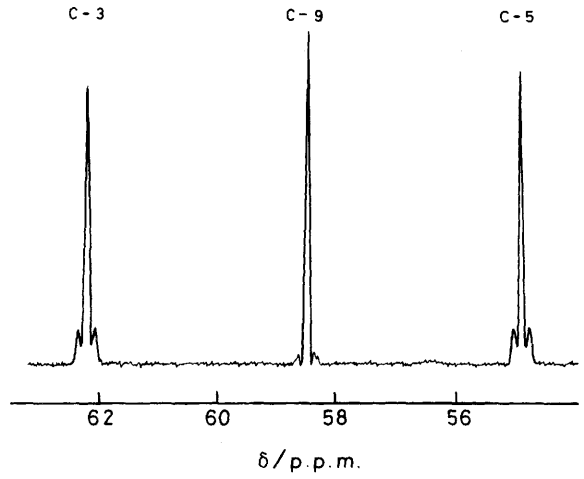


Figure 2. Part of the resolution enhanced proton-noise decoupled ^{13}C n.m.r. spectrum of retronecine (**3**) hydrochloride enriched with [1-*amino*-^{15}N,1-^{13}C]putrescine (**11**) dihydrochloride

is the average enrichment due to ^{13}C at each labelled position of putrescine (**11**)].

Moreover, the resolution-enhanced spectrum of retronecine hydrochloride (Figure 2) showed the presence of doublets at δ 54.9 (*J* 4.5 Hz) and 62.2 p.p.m. (*J* 5.0 Hz) with enrichment factors of 0.2 ± 0.05 and 0.25 ± 0.05% ^{13}C respectively. [The rest of the ^{13}C enriched material associated with C-3 and C-5 yields singlets coincident with natural abundance material, owing to cleavage of the ^{13}C–^{15}N bond in the biosynthetic pathway. The labelled species derived from a single molecule of putrescine (**11**) are shown in Scheme 4.] The different values observed for the two coupling constants suggests that the two carbons are not coupled to each other and that ^{13}C–^{15}N species are present in the retronecine. The presence of these species was confirmed by observation of the 36.5 MHz $^{15}N\{^1H\}$ n.m.r. spectrum of retronecine hydrochloride taken in deuterium oxide. This showed ^{13}C–^{15}N satellites (*J ca.* 5 Hz) in addition to the natural abundance signal at δ 311.2 p.p.m. upfield from external nitromethane. The extra coupling around C-9 (Figure 2) (*J ca.* 6 Hz) is probably due to coupling with C-8 arising from the combination of two labelled molecules of putrescine (**11**).* The approximately equal enrichment of C-3 and C-5 of retronecine with ^{13}C–^{15}N species [as in (**12**)] provides strong evidence for the involvement of a symmetrical C_4–N–C_4 intermediate in retronecine biosynthesis. Similar results were obtained by Grue-Sørensen and Spenser for retronecine obtained from a mixture of pyrrolizidine alkaloids in *Senecio vulgaris* plants.[9]

Evidence that this later symmetrical intermediate is *N*-(4-aminobutyl)-1,4-diaminobutane (homospermidine) has been provided by the intact incorporation of [1,9-^{14}C]-^{15} and [1,9-$^{13}C_2$]-homospermidine[16] into retronecine. The conversion of homospermidine into 1-hydroxymethylpyrrolizidine has also been achieved recently using enzymes and physiological conditions.[17]

Experimental

General.—M.p.s were measured with a Kofler hot-stage apparatus. Organic solutions were dried with anhydrous Na_2SO_4, and solvents were evaporated off under reduced pressure below 40 °C. ^{13}C N.m.r. spectra were obtained on a Varian XL-100 spectrometer operating at 25 MHz, or on a Bruker WH-360 spectrometer at 90 MHz. Mass spectra were obtained with A.E.I. MS 12 or MS 902 spectrometers. All ^{13}C-labelled compounds were purchased from B.O.C. Prochem Ltd., London. Radioactivity was measured with a Philips liquid scintillation analyser using toluene–methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were recrystallized to constant specific activity and were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

[1,4-$^{13}C_2$]-1,4-*Diaminobutane* (**8**) (*Putrescine*) *Dihydrochloride.*—A solution of potassium [^{13}C]cyanide (93 atom % ^{13}C) (1.0 g, 15.4 mmol) in water (5 ml) was added to 1,2-dibromoethane (0.96 g, 5.1 mmol) in ethanol (100 ml), and the mixture was heated at reflux for 4 h. The cooled solution was concentrated and the residue was taken up in water (50 ml) and extracted continuously with chloroform for 24 h. The chloroform extracts were dried, filtered, and concentrated to an oil. Purification was carried out by column chromatography on silica gel, and elution with benzene–methanol (100:1). Solid [1,4-$^{13}C_2$]succinonitrile (230 mg, 57%) was crystallized from benzene; m.p. 53—55 °C (lit.,[18] 57 °C); $\nu_{max.}$ (KBr) 2 255 cm^{-1}; $\delta_H(CD_3OD)$ 2.90 (s); δ_C 117.4 p.p.m. (s) (Found: M^+ 82.0441. Calc. for $^{12}C_2{}^{13}C_2H_4N_2$: *M* 82.0441). 1M-Borane in dry tetrahydrofuran (25 ml) was added to a solution of [1,4-$^{13}C_2$]succinonitrile (225 mg, 2.74 mmol) in dry tetrahydrofuran (25 ml) and the solution was heated at reflux for 20 h. Dry ethanol (50 ml) was added to the cooled solution. After 1 day, dry HCl gas was passed through the solution. The solid dihydrochloride of [1,4-$^{13}C_2$]putrescine (**8**) (282 mg, 63%) was filtered off and crystallized from aqueous ethanol; $\delta_H(D_2O)$ 1.8 (2 H, s) and 3.1 (0.15 H, s, + 1.85 H, d, $J_{^{13}C^1H}$ 160 Hz); $\delta_C\{^1H\}$ (D_2O) 39.8 p.p.m. (s).

1,4-*Diamino*[2,3-$^{13}C_2$]*butane* (**9**) *Dihydrochloride.*—In a similar fashion, a solution of potassium cyanide (2.0 g, 31 mmol) in water (10 ml) was treated with 1,2-dibromo[1,2-$^{13}C_2$]ethane (81% $^{13}C_2$, 18% $^{13}C^{12}C$, and 1% $^{12}C_2$ species) (1 g, 5.3 mmol) to afford [2,3-$^{13}C_2$]succinonitrile (217 mg, 53%); m.p. 53—54 °C (lit.,[18] 57 °C); $\nu_{max.}$(KBr) 2 260 cm^{-1}; $\delta_C\{^1H\}$ (CD_3OD) 14.7 p.p.m. (s) (Found: M^+ 82.0443. Calc. for $^{12}C_2{}^{13}C_2H_4N_2$: *M* 82.0441). Reduction of the [2,3-$^{13}C_2$]succinonitrile with borane in tetrahydrofuran, followed by treatment with HCl gas, and recrystallization from aqueous ethanol gave the dihydrochloride of [2,3-$^{13}C_2$]putrescine (**8**) (251 mg, 57%); $\delta_H(D_2O)$ 1.8 (0.2 H, s + 1.8 H, d, $J_{^{13}C^1H}$ 135 Hz) and 3.2 (2 H, s); $\delta_C\{^1H\}$ (D_2O) 24.7 p.p.m. (s).

1,4-*Diamino*[1-^{13}C]*butane* (**10**) *Dihydrochloride.*—Benzyl chloroformate (3.0 ml, 21 mmol) was added slowly with stirring during 30 min to a solution of 3-bromopropylamine hydrobromide (4.2 g, 19.2 mmol) in dichloromethane (50 ml) and triethylamine (7 ml) at 0 °C. The mixture was allowed to warm to room temperature and stirring was continued for 3 h. The mixture was washed with water, dilute HCl, and water (each 50 ml). The organic layer was dried, filtered, and concentrated to a colourless syrup (5.2 g). Purification by column chromatography on silica gel and elution with benzene afforded *N-benzyloxycarbonyl-3-bromopropylamine* as an oil (4.1 g, 79%); R_F 0.52 (benzene–methanol, 9:1); $\nu_{max.}$(CCl_4) 3 470, 1 732, 1 510, 1 230, and 700 cm^{-1}; $\delta_H(CDCl_3)$ 2.03 (2 H, m), 3.33 (4 H, m), 5.07 (2 H, s), and 7.27 (5 H, s); *m/z* 273 (M^+), 271, 108, 91, 79, 77, and 65 (Found: C, 48.4; H, 5.3; Br, 29.6; N, 5.1. $C_{11}H_{14}BrNO_2$ requires C, 48.54; H, 5.19; Br, 29.36; N, 5.15%). Dry, powdered potassium [^{13}C]cyanide (93 atom % ^{13}C; 1.0 g, 15 mmol) was added to a solution of *N*-benzyloxycarbonyl-3-bromopropylamine (3.4 g,

* See footnote * on p. 102.

12.5 mmol) in hexamethylphosphoramide (HMPA) (20 ml) containing 18-crown-6 (0.53 g, 2 mmol).[19] The mixture was heated at 80 °C for 5 h, then cooled and poured into water (100 ml), and the aqueous solution extracted with ethyl acetate (5 × 100 ml). The organic layers were combined, washed with water (10 × 10 ml), dried, filtered, and concentrated to yield 4-*benzyloxycarbonylamino*[1-^{13}C]*butanenitrile* as an oil (1.94 g, 71%); $\nu_{max.}$(film) 3 340, 2 245, 1 720, 1 520, 1 260, and 700 cm^{-1}. Unlabelled material: δ_H($CDCl_3$) 1.85 (2 H, m), 2.28 (2 H, m), 3.22 (2 H, m), 5.10 (2 H, s), and 7.30 (5 H, s); *m/z* 218 (M^+), 191, 173, 108, 107, and 91. Labelled material: δ_C($CDCl_3$) 118.9 p.p.m. (s) (Found: M^+ 219.1088. $^{12}C_{11}{}^{13}C_1H_{14}N_2O_2$ requires *M*, 219.1087).

A solution of 4-benzyloxycarbonylamino[1-^{13}C]butanenitrile (1.9 g, 8.7 mmol) in ethanol–0.1M-HCl (1:1; 100 ml) was hydrogenated at 10 atm in the presence of 10% Pd/C (1 g) for 18 h. The catalyst was filtered off with Celite, and the filtrate was concentrated to give a white solid (1.3 g). Recrystallization from aqueous ethanol gave [1-^{13}C]putrescine (**10**) dihydrochloride (0.91 g, 65%); δ_H(D_2O) 1.8 (2 H, s) and 3.1 (1.1 H, s + 0.9 H, d, $J_{^{13}C^1H}$ 160 Hz); $\delta_C\{^1H\}$ (D_2O) 39.8 p.p.m. (s).

1,4-*Diamino*[1-amino-^{15}N,1-^{13}C]*butane* (**11**) *Dihydrochloride*.—Dry, powdered potassium [^{13}C,^{15}N]cyanide (90.6 atom % ^{13}C, 99.4 atom % ^{15}N; 0.10 g, 1.5 mmol) was added to a solution of *N*-benzyloxycarbonyl-3-bromopropylamine (0.34 g, 1.25 mmol) in HMPA (2 ml) containing 18-crown-6 (50 mg). This mixture was heated at 80 °C for 4 h. Similar work-up to that used in the previous synthesis gave 4-*benzyloxycarbonylamino*-N-[1-^{15}N,1-^{13}C]*butanenitrile* as an oil (162 mg, 59%); δ_C($CDCl_3$) 119.0 p.p.m. (d, *J* 17 Hz) (Found: M^+ 220.1059. $^{12}C_{11}{}^{13}C_1H_{14}{}^{14}N^{15}NO_2$ requires *M* 220.1057). This nitrile was hydrogenated as described above in ethanolic HCl, and recrystallization of the product from aqueous ethanol gave [1-*amino*-^{15}N,1-^{13}C]putrescine (**11**) dihydrochloride (70 mg, 58%); δ_H(D_2O) 1.8 (4 H, br s), and 3.1 (2.2 H, br s + 1.8 H, d, $J_{^{13}C^1H}$ 140 Hz); $\delta_C\{^1H\}$ (D_2O) 39.6 p.p.m. (d, *J* 5.1 Hz).

Feeding Methods.—*Senecio isatideus* plants were propagated from stem cuttings and grown on in a standard compost. The age and number of plants used in each experiment is shown in Table 1. [1,4-^{14}C]Putrescine dihydrochloride (10 μCi) (Amersham International) was added to each ^{13}C-labelled precursor. The precursors were dissolved in sterile water, and drops of these solutions were introduced into the xylems of the plants through stem punctures made with a sterile needle. Each precursor was pulse fed on alternate days. One week after administration of the precursor had ceased, the plants were harvested and retrorsine (**1**) was isolated as described previously.[7] Retrorsine was recrystallized to constant specific activity from acetone, m.p. 215—216 °C (lit.,[7] 216—217 °C). Radioscans of silica gel G t.l.c. plates of 0.25-mm thickness developed with chloroform–methanol–conc. ammonia (85:14:1) showed one radioactive band, coincident with authentic unlabelled retrorsine (**1**) at R_F 0.35. Alkaloids were visualized by oxidation with *o*-chloranil, followed by treatment with Ehrlich's reagent.[20]

Hydrolysis of Retrorsine (**1**).—A mixture of retrorsine (60 mg, 0.17 mmol) and barium hydroxide octahydrate (140 mg, 0.444 mmol) were heated at reflux in water (2 ml) for 2 h. The cooled solution was saturated with solid carbon dioxide and barium carbonate was filtered off. The filtrate was basified with solid sodium carbonate and extracted continuously with diethyl ether for 2 days. The ether extracts were dried, filtered, and concentrated to a residue, which gave prisms of retronecine (**3**) (20 mg, 76%) from acetone. Retronecine was converted into its hydrochloride and recrystallized to constant specific activity, m.p. 164—165 °C (lit.,[21] 164 °C); δ_C[unlabelled material in (D_2O)] 137.4 (C-1), 122.1 (C-2), 79.6 (C-8), 70.1 (C-7), 62.2 (C-3), 58.5 (C-9), 54.9 (C-5), and 35.9 p.p.m. (C-6).

The aqueous layer from the ether extraction was acidified to pH 1 with conc. HCl, and was extracted continuously with diethyl ether for 2 days. The ether extracts were dried, filtered, and evaporated to give a residue, which afforded needles of isatinecic acid (**2**) (30 mg, 82%) from ethyl acetate. The acid was recrystallized to constant specific activity, m.p. 145—147 °C (lit.,[22] 148 °C).

The results obtained from feeding the ^{13}C-labelled putrescine precursors (**8**)—(**11**) are summarized in Tables 1 and 2.

The ^{13}C n.m.r. spectra of retrorsine (**1**) enriched with ^{13}C after feeding experiments with the putrescine precursors (**9**)—(**11**) allowed the complete assignment of the ^{13}C n.m.r. spectrum of retrorsine: δ 175.6 (C-11), 167.4 (C-16), 136.7 (C-2), 134.6 (C-20), 132.6 (C-15), 131.4 (C-1), 81.6 (C-12), 77.5 (C-8), 75.1 (C-7), 67.0 (C-18), 62.9 (C-3), 61.0 (C-9), 53.0 (C-5), 38.0 (C-14), 35.7 (C-13), 34.7 (C-6), 15.0 (C-21), and 11.6 p.p.m. (C-19).

Acknowledgements

We are grateful to the S.E.R.C. for a Research Assistantship (to H. A. K.) and for use of the high-field n.m.r. service.

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Received 11*th May* 1984; *Paper* 4/760