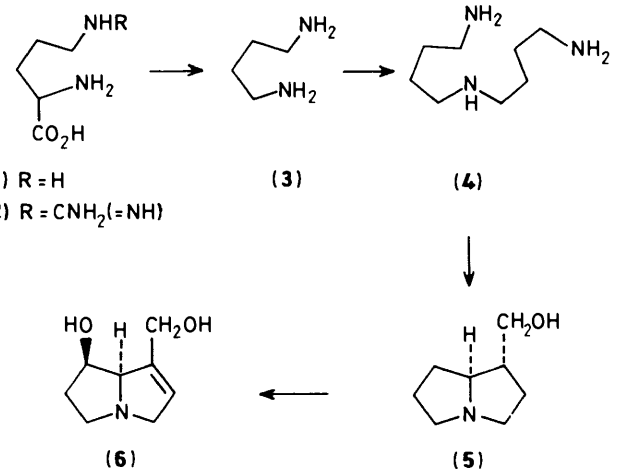
Pyrrolizidine Alkaloid Biosynthesis. Incorporation of ^{13}C-Labelled Precursors into Rosmarinine

Henry A. Kelly and David J. Robins *
Department of Chemistry, University of Glasgow, Glasgow G12 8QQ

Biosynthesis of the rosmarinecine (**8**) portion of the pyrrolizidine alkaloid rosmarinine (**7**) has been investigated in *Senecio pleistocephalus* plants using ^{13}C-labelled precursors. These plants were fed with [1-^{13}C]putrescine dihydrochloride (**12**) and [2,3-$^{13}C_2$]putrescine (**13**) dihydrochloride, and the labelling patterns in the biosynthetically derived rosmarinine were established by ^{13}C n.m.r. spectroscopy. Two molecules of putrescine are incorporated to about the same extent into rosmarinecine (**8**). Incorporation of [1-*amino*-^{15}N,1-^{13}C]putrescine (**14**) dihydrochloride into rosmarinine produced a labelling pattern which was consistent with conversion of the two putrescine molecules into a C_4–N–C_4 symmetrical intermediate. This intermediate was identified as homospermidine (**4**) by feeding [1,9-$^{13}C_2$]homospermidine (**15**) trihydrochloride to *S. pleistocephalus* plants. Intact incorporation of this precursor was demonstrated by observation of two enriched ^{13}C n.m.r. signals for C-8 and C-9 of rosmarinine.

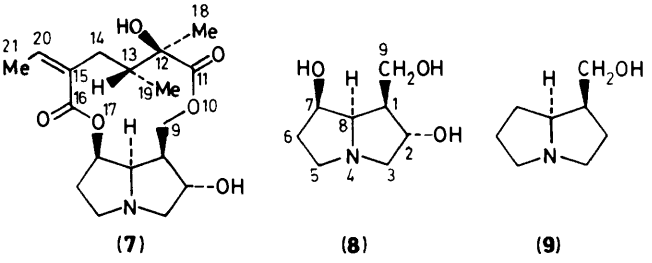
Retronecine (**6**) is the most common base component (necine) found in the pyrrolizidine alkaloids.[1] By using radioactive precursors it has been shown that retronecine is formed biosynthetically from two molecules of ornithine (**1**),[2-5] or arginine (**2**)[4,5] (only the L-enantiomers are utilised[6]) *via* putrescine (**3**)[3,5] (Scheme 1). However, the labelling patterns



Scheme 1.

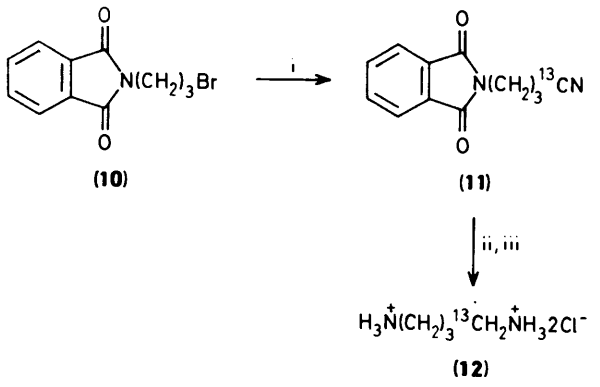
obtained by degradation to establish the distribution of the ^{14}C and ^{3}H labels in these feeding experiments were incomplete.[5,7] Complete labelling patterns were first obtained by ^{13}C n.m.r. spectroscopy after feeding ^{13}C-labelled precursors to produce labelled alkaloids in *Senecio isatideus*.[8] These labelling patterns indicated that two molecules of putrescine are incorporated to about the same extent into each molecule of retronecine. Feeding experiments with [1-*amino*-^{15}N,1-^{13}C]putrescine demonstrated that a later C_4–N–C_4 intermediate with C_{2v} symmetry is involved in retronecine biosynthesis.[7,9] This later intermediate was shown to be 1,6,11-triazaundecane (homospermidine) (**4**) by carrying out feeding experiments with homospermidine labelled with ^{14}C[10] and with ^{13}C.[11] Additional support for the involvement of homospermidine in pyrrolizidine alkaloid biosynthesis was provided by the conversion of homospermidine (**4**) into the 1-hydroxymethylpyrrolizidine (±)-trachelanthamidine (**5**) using enzymes and physiological conditions.[12] Furthermore, ^{14}C-labelled (±)-trachelanthamidine was recently shown to be a good precursor for retronecine (**6**) in *S. riddellii*[13] and *S. isatideus*.[14]

Investigation of the biosynthesis of necines has been confined to retronecine (**6**), although a wide range of necines has been isolated.[1] We desired to widen the scope of the biosynthetic studies in this area. The major alkaloidal constituent of *Senecio pleistocephalus* is rosmarinine (**7**), which contains rosmarinecine (**8**) as the base component. We recently showed that the diastereoisomeric 1-hydroxymethylpyrrolizidine (±)-isoretronecanol (**9**) is a much better precursor for rosmarinecine than (±)-trachelanthamidine (**5**) in direct contrast to retronecine biosynthesis.[14] We therefore decided it was necessary to investigate the early steps of the biosynthetic pathway to rosmarinecine.



Results and Discussion

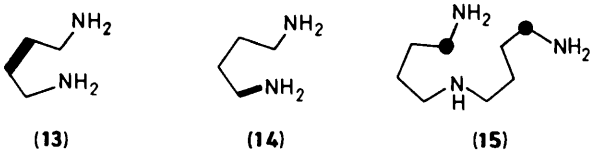
Extraction from *Senecio pleistocephalus* S. Moore plants gave one major crystalline alkaloid, m.p. 204 °C. This alkaloid gave a positive test with Dragendorff's reagent,[15] whereas treatment of the alkaloid with *o*-chloranil followed by Ehrlich's reagent[16] (without warming the t.l.c. plate) showed that there was no pyrrolic material present. Thus the alkaloid does not contain 1,2-unsaturation as present in alkaloids containing retronecine (**6**). High resolution mass spectrometry and analytical data established the molecular formula of the alkaloid as $C_{18}H_{27}NO_6$. Only two known pyrrolizidine alkaloids containing saturated necines, hygrophylline and rosmarinine, possess this molecular formula.[1] The ^{1}H and ^{13}C n.m.r. spectroscopic data for the alkaloid were closely similar to those reported for rosmarinecine (**7**).[17] The identity was confirmed by non-depression of a mixed m.p. with an authentic sample of rosmarinine, and by *X*-ray crystal structure analysis.[18] Because of our intention to study the biosynthesis of rosmarinine using ^{13}C-labelled precursors, assignment of the ^{13}C n.m.r. spectrum



Scheme 2. *Reagents:* i, $Na^{13}CN$; ii, H_2–PtO_2, AcOH; iii, HCl

of rosmarinine (**7**) with the natural abundance of the isotope was performed carefully on the basis of a distortionless enhancement by polarisation transfer (DEPT) experiment and heteronuclear (^{13}C–^{1}H) chemical shift correlation spectroscopy. These assignments were in agreement with literature values.[17]

Preliminary feeding experiments on *Senecio pleistocephalus* plants were carried out with [1-^{13}C]putrescine dihydrochloride (**12**). This was prepared by a modified procedure,[9] involving treatment of *N*-(3-bromopropyl)phthalimide (**10**) with sodium [^{13}C]cyanide followed by catalytic hydrogenation of the nitrile and acid hydrolysis (Scheme 2). Radioactive [1,4-^{14}C]-putrescine dihydrochloride was added to this ^{13}C-labelled precursor (**12**), and feeding experiments were carried out with *S. pleistocephalus* plants of various ages and by two different feeding techniques. The wick method gave higher incorporations than those obtained by pulsed feeding of the precursor mixture into the xylems of the plants through stem punctures.[5]



The highest incorporation of the precursor (**12**) was obtained by feeding freshly rooted cuttings on each day for one week by the wick method. One week later, the plants were harvested, and rosmarinine (**7**) was isolated and recrystallised to constant specific radioactivity* of 22% per C_4 unit. The ^{13}C-{^{1}H} n.m.r. spectrum of the ^{13}C-labelled rosmarinine in deuteriochloroform (Figure 1) was compared with that of unlabelled material run under the same conditions. Four enhanced signals were observed, corresponding to C-3, C-5, C-8, and C-9 of rosmarinine, with enrichment factors† for each position of 12.0, 12.2, 10.0, and 10.0 (all ± 1.0)% ^{13}C, respectively. The average enrichment factor for each labelled site was 11.1% ^{13}C, and the estimated ^{13}C specific incorporation was 11.1 × 2/91 × 100 = 24.4% per C_4 unit of putrescine, where 91/2 atom % ^{13}C was the average enrichment at each labelled position of putrescine

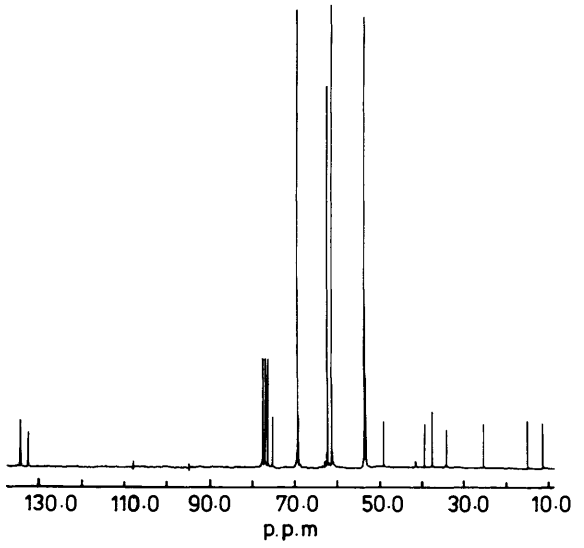


Figure 1. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (**7**) in $CDCl_3$ enriched with [1-^{13}C]putrescine dihydrochloride (**12**)

(**12**). Some broadening at the base of the four enriched signals in the ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine was noticeable (Figure 1), probably due to geminal coupling (*i.e.* C-3 with C-5 or C-8, and C-8 with C-9 of rosmarinecine) arising from the combination of two ^{13}C-labelled putrescine (**12**) units as a consequence of the extremely high incorporations obtained.[9]

A ^{13}C–^{13}C doubly labelled precursor (**13**) was used next to provide a complementary labelling pattern in rosmarinine. This experiment also has the advantage that smaller amounts of precursor can be fed, and lower enrichments of labelled sites may be identified by observation of ^{13}C–^{13}C couplings in the ^{13}C-{^{1}H} n.m.r. spectrum of the alkaloid produced. The dihydrochloride of [2,3-$^{13}C_2$]putrescine (**13**) was prepared from [1,2-$^{13}C_2$]-1,2-dibromoethane by treatment with sodium cyanide, followed by reduction of the dinitrile, and acidification of the product.[9] This material, together with [1,4-^{14}C]-putrescine dihydrochloride was fed to one well-established *Senecio pleistocephalus* plant, and a ^{14}C specific incorporation* of 2.4% for the rosmarinine produced was measured. The ^{13}C-{^{1}H} n.m.r. spectrum of the labelled rosmarinine (Figure 2) showed a pair of doublets at δ 49.1 (*J* 35.2 Hz) and 69.1 p.p.m. (*J* 35.2 Hz) corresponding to C-1 and C-2, and another pair of doublets at δ 34.4 (*J* 35.4 Hz) and 75.3 p.p.m. (*J* 35.5 Hz) corresponding to C-6 and C-7 of rosmarinine, respectively. The enrichment factors† for the four labelled sites C-1, C-2, C-6, and C-7 in rosmarinine were 1.69, 1.93, 1.76, and 1.63 (all ± 0.1)% ^{13}C, respectively. The average enrichment factor for each labelled site was 1.75% ^{13}C, which gave an estimated ^{13}C specific incorporation of 1.75 × 1/81 × 100 = 2.2% ^{13}C, where the average enrichment at each labelled position of putrescine (**13**) was 81 atom % ^{13}C. The four labelled sites in rosmarinine (Figure 2) display nearly equal enrichment factors, supporting the theory initially proposed for retronecine biosynthesis[8] that two molecules of putrescine combine to form a later C_4–N–C_4 intermediate in the biosynthesis of rosmarinine (**8**).

As with retronecine biosynthesis,[7,9] evidence for this later intermediate with C_{2v} symmetry was obtained by using the [^{13}C–^{15}N] doubly labelled precursor (**14**). A sample of [1-*amino*-^{15}N,1-^{13}C]putrescine (**14**) dihydrochloride was prepared by treatment of the benzyloxycarbonyl derivative of 3-bromopropylamine with potassium [^{13}C–^{15}N]cyanide followed by catalytic hydrogenation and acidification of the diamine. The feeding experiment was carried out as usual with one well

* Specific ^{14}C incorporation per C_4 unit for a putrescine precursor is calculated from [(molar activity of rosmarinine (**7**) × 1/2)/(molar activity of precursor)] × 100%

† The enrichment factor for each labelled site in rosmarinine (**7**) 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%

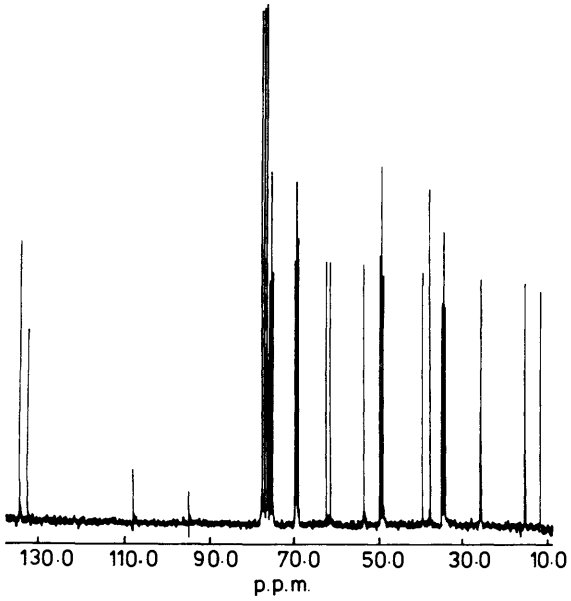


Figure 2. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (**7**) in $CDCl_3$ enriched with [2,3-$^{13}C_2$]putrescine (**13**) dihydrochloride

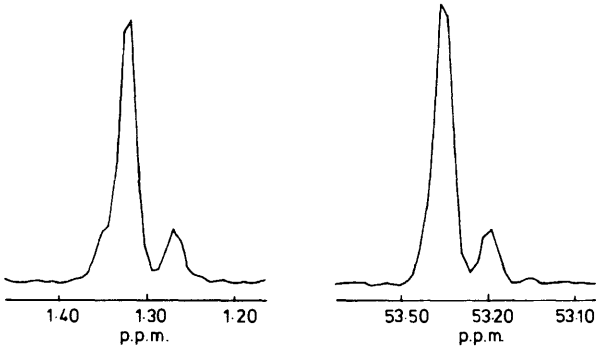


Figure 3. Part of the 50 MHz resolution-enhanced ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine (**7**) in $CDCl_3$ enriched with [1-*amino*-^{15}N,1-^{13}C]putrescine (**14**) dihydrochloride

established *Senecio pleistocephalus* plant, and rosmarinine was isolated with a ^{14}C specific incorporation* of 1.1% per C_4 unit. The ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine showed four enriched sites. Enrichment factors† of 0.3 ± 0.05% ^{13}C were observed for C-3 and C-9 at δ 61.3 and 62.2 p.p.m. respectively, and values of 0.4 ± 0.05% ^{13}C were measured for the signals due to C-5 and C-8 at δ 53.5 and 69.3 p.p.m., respectively. The average enrichment factor was 0.35% ^{13}C for each labelled site, which corresponded to a specific ^{13}C incorporation per C_4 unit of 0.35 × 2/90.6 × 100 = 0.77% ^{13}C, where 90.6/2 atom % ^{13}C was the average enrichment of ^{13}C at each labelled position of putrescine (**14**). Additional coupling was observed around the signals for C-3 and C-5 of rosmarinine in the resolution enhanced ^{13}C n.m.r. spectrum (Figure 3), although the doublets were not fully resolved. Enrichment factors and coupling constants were estimated to be 0.15 ± 0.05 (*J* 4 Hz) for C-3 and 0.2 ± 0.05% ^{13}C (*J* 2—3 Hz) for C-5. Although there is some uncertainty in the values for these coupling constants, it appears that they have different values. This indicates that the two carbons are not coupled to each other and that ^{13}C–^{15}N species are present. The observation of two ^{13}C–^{15}N doublets of about

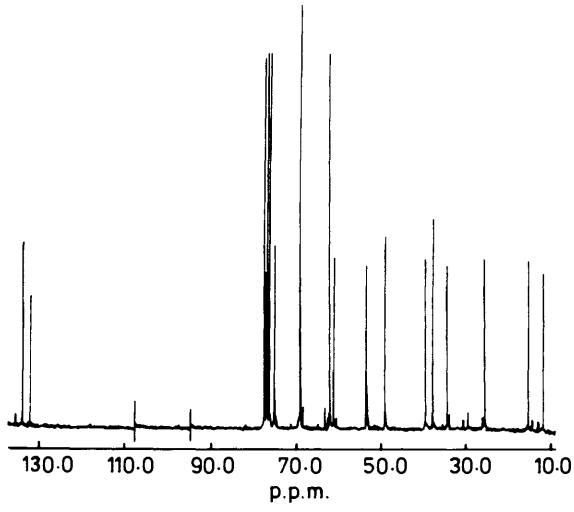


Figure 4. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (**7**) in $CDCl_3$ enriched with [1,9-$^{13}C_2$]homospermidine (**15**) trihydrochloride

equal intensity associated with C-3 and C-5 of rosmarinine is consistent with the existence of a later intermediate with C_{2v} symmetry in the biosynthetic pathway to rosmarinecine. This intermediate was shown to be homospermidine (**4**) in retronecine biosynthesis after demonstration of the intact incorporation of [1,9-^{14}C][10] and [1,9-$^{13}C_2$]homospermidine.[11]

The dihydrochloride of [1,9-$^{13}C_2$]homospermidine (**15**) was synthesized by reaction of benzylamine with two equivalents of 4-chloro-[1-^{13}C]butanenitrile followed by catalytic hydrogenation and acidification.[11] The ^{14}C-labelled material was prepared in the same way from 4-chloro-[1-^{14}C]butanenitrile. The mixture of precursors was fed to one *Senecio pleistocephalus* plant, and rosmarinine was produced with a ^{14}C specific incorporation of 1.4%. Only two enriched signals were observed in the ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine at δ 62.2 and 69.3 p.p.m. due to C-9 and C-8, respectively (Figure 4). Enrichment factors† of 1.58 and 1.50 (both ± 0.1)% ^{13}C were measured for C-8 and C-9. The ^{13}C specific incorporation is therefore 1.54 × 1/96 × 100 = 1.6%, where 96 atom % ^{13}C was the average enrichment at each labelled site in homospermidine (**15**). No doublets were observed around the signals for C-8 and C-9 in the resolution enhanced ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine, indicating that the geminal coupling constant between C-8 and C-9 of rosmarinine is zero (a value of 6 Hz was observed for retronecine).[11] Nevertheless, it is clear that only the signals for C-8 and C-9 of rosmarinine show enrichment with ^{13}C; no enhancement above natural abundance was observed for any of the other signals in the ^{13}C{^{1}H} n.m.r. spectrum. Breakdown of the homospermidine prior to formation of rosmarinecine (**8**) could not be detected.

The biosynthesis of rosmarinecine (**8**) takes place from two molecules of putrescine *via* homospermidine in *Senecio pleistocephalus* plants. The pathway to rosmarinecine then proceeds *via* isoretronecanol (**9**), whereas retronecine (**6**) is formed from trachelanthamidine (**5**). Further information about the point at which the two pathways diverge may be obtained by establishing the stereochemistry of the enzymic processes involved in rosmarinecine biosynthesis. These details are already known for retronecine from the use of the enantiomeric [1-^{2}H]-[19] and [2-^{2}H]-putrescines.[20]

Experimental

General.—M.p.s were measured with a Kofler hot-stage apparatus. ^{13}C N.m.r. spectra were obtained on a Bruker WP-

* See corresponding footnote on p. 178.
† See corresponding footnote on p. 178.

200SY spectrometer operating at 50 MHz. All ^{13}C-labelled compounds were purchased from B.O.C. Prochem Ltd., London. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using toluene–methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were recrystallised to constant specific radioactivity and they were counted in duplicate. A Panax thin layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

1,4-Diamino[1-^{13}C]*butane Dihydrochloride* (**12**).—Dry, powdered sodium [^{13}C]cyanide (1 g, 20 mmol, 91 atom % ^{13}C) was added to a solution of *N*-(3-bromopropyl)phthalimide (**10**) (4.47 g, 16.6 mmol) in dry dimethyl sulphoxide (50 ml). The mixture was stirred at 90 °C for 2 h, and then left overnight at room temperature. Diethyl ether (300 ml) was added, and the mixture was washed with water (6 × 50 ml), and brine (3 × 50 ml). The organic layer was dried (Na_2SO_4), filtered, and concentrated to yield 4-phthalimido[1-^{13}C]butanenitrile (**11**) as an oil (1.75 g, 49%); $\nu_{max.}$(film) 2 200, 1 775, and 1 715 cm^{-1}; $\delta_H(CDCl_3)$ 2.09 (2 H, t, *J* 7.2 Hz), 2.4 (2 H, m), 3.82 (2 H, t, *J* 7.2 Hz), and 7.75 (4 H, m); δ_C-{^1H}($CDCl_3$) 118.6 p.p.m. (s); *m/z* 216 (M^+), 160, 133, 105, 104, and 76.

4-Phthalimido[1-^{13}C]butanenitrile (**11**) (0.5 g, 2.3 mmol) was added to a suspension of platinum oxide (75 mg) in glacial acetic acid (12 ml), and the mixture was hydrogenated at atmospheric pressure for 4 h. The mixture was filtered, and the filtrate was concentrated to give crude 1-amino-4-phthalimido[1-^{13}C]-butane (450 mg, 88%). The crude product was hydrolysed by heating it at reflux in concentrated HCl (15 ml) for 20 h. Precipitated phthalic acid was removed by filtration after cooling the reaction product to 0 °C. The filtrate was evaporated to dryness, and the residue was recrystallised from aqueous ethanol to yield 1,4-diamino[1-^{13}C]butane dihydrochloride (**12**) (276 mg, 83%); $\delta_H(D_2O)$ 1.79 (4 H, br s) and 3.08 (2.2 H, br s + 1.8 H, d, $J_{^{13}C^1H}$ 150 Hz); δ_C-{^1H}(D_2O) 39.6 p.p.m. (s).

The dihydrochlorides of 1,4-diamino[2,3-$^{13}C_2$]butane (**13**),[9] 1,4-diamino[1-*amino*-^{15}N,1-^{13}C]butane (**14**),[9] and the trihydrochlorides of [1,9-$^{13}C_2$]homospermidine (**15**), and [1,9-^{14}C]-homospermidine were prepared as described.[11]

Feeding Methods.—*Senecio pleistocephalus* S. Moore plants were obtained from the Royal Botanic Garden, Edinburgh (No. 710277). They were propagated from stem cuttings and grown in a standard compost in a greenhouse. A sample of [1,4-^{14}C]putrescine dihydrochloride (5 or 10 μCi) (Amersham International) was added to each ^{13}C-labelled putrescine precursor, and [1,9-^{14}C]homospermidine trihydrochloride (20 μCi) was added to the [1,9-^{13}C]homospermidine (**15**) trihydrochloride. The precursor mixtures were dissolved in sterile water, and fed by the Wick method to one plant for each experiment. The precursors were fed on successive days for one week. One week after administration of the precursors was complete, the plants were harvested and rosmarinine (**7**) was isolated as for other pyrrolizidine alkaloids[5] in 0.15% yield, based on the weight of fresh plant material. Rosmarinine was recrystallised to constant specific radioactivity from dichloromethane–acetone, m.p. 204 °C (decomp.), (lit.,[17] 202—204 °C); $[\alpha]_D^{14}$ −85.6 °C (*c* 1, MeOH) (lit.,[17] $[\alpha]_D^{24}$ −85.3 °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 rosmarinine (**7**) at R_F 0.30. Alkaloids were visualised by the modified Dragendorff reagent.[15] Rosmarinine; $\delta_C(CDCl_3)$, 11.6 (C-19), 15.1 (C-21), 25.6 (C-18), 34.4 (C-6), 37.8 (C-13), 39.5 (C-14), 49.1 (C-1), 53.5 (C-5), 61.3 (C-3), 62.2 (C-9), 69.1 (C-2), 69.3 (C-8), 75.3 (C-7), 77.5 (C-12), 132.7 (C-15), 134.4 (C-20), 167.5 (C-16), and 180.6 p.p.m. (C-11).

Acknowledgements

We thank the S.E.R.C. for a Research Assistantship (to H. A. K.). We are grateful to the Staff of the Royal Botanic Garden, Edinburgh for identifying and supplying *Senecio pleistocephalus*. We thank Dr. D. S. Rycroft (Glasgow) for running the n.m.r. spectra.

References

1 D. J. Robins, *Fortschr. Chem. Org. Naturst.*, 1982, **41**, 115; D. J. Robins, *Nat. Prod. Rep.*, 1984, **1**, 235; *ibid.*, 1985, **2**, 213; *ibid.*, 1986, **3**, in the Press.
2 E. Nowacki and R. U. Byerrum, *Life Sci.*, 1962, **1**, 157; C. A. Hughes, R. Letcher, and F. L. Warren, *J. Chem. Soc.*, 1964, 4974.
3 W. Bottomley and T. A. Geissman, *Phytochemistry*, 1964, **3**, 357.
4 N. M. Bale and D. H. G. Crout, *Phytochemistry*, 1975, **14**, 2617.
5 D. J. Robins and J. R. Sweeney, *J. Chem. Soc., Perkin Trans. 1*, 1981, 3083.
6 D. J. Robins, and J. R. Sweeney, *Phytochemistry*, 1983, **22**, 457.
7 G. Grue-Sorensen and I. D. Spenser, *Can. J. Chem.*, 1982, **60**, 643.
8 H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 146.
9 H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 554; *J. Chem. Soc., Perkin Trans. 1*, 1985, 101.
10 H. A. Khan and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1985, 619.
11 J. Rana, and D. J. Robins, *J. Chem. Res.*, 1983, (*S*), 146.
12 D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1982, 1289.
13 J. Rana and E. Leete, *J. Chem. Soc., Chem. Commun.*, 1985, 1742.
14 E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1986, 250.
15 R. Munier and M. Macheboef, *Bull. Soc. Chim. Biol.*, 1951, **33**, 846.
16 H. J. Huizing, F. DeBoer, and T. M. Malingrè, *J. Chromatogr.*, 1980, **195**, 407; R. J. Molyneux and J. N. Roitman *ibid.*, 1980, **195**, 412.
17 J. N. Roitman, *Aust. J. Chem.*, 1983, **36**, 1203.
18 A. A. Freer, H. A. Kelly, and D. J. Robins, *Acta Crystallogr. Sect. C*, in the press.
19 G. Grue-Sorensen and I. D. Spenser, *J. Am. Chem. Soc.*, 1983, **105**, 7401; J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1984, 517.
20 E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1985, 1450.

Received 25*th February* 1986; *Paper* 6/399