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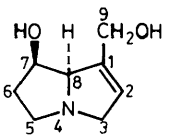
Biosynthesis of Pyrrolizidine Alkaloids

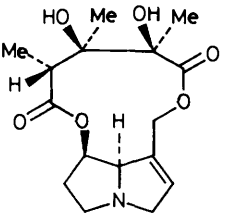
By David J. Robins
DEPARTMENT OF CHEMISTRY, UNIVERSITY OF GLASGOW, GLASGOW G12 8QQ

1 Introduction

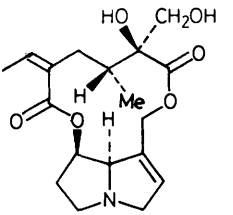
Pyrrolizidine alkaloids constitute a large class of natural products with over 200 known examples. They have a widespread occurrence and are found mainly in *Senecio* spp. (Compositae), *Crotalaria* spp. (Leguminosae), and *Heliotropium* spp. (Boraginaceae).[1] It is estimated that 3% of the World's flowering plants may contain these alkaloids. Pyrrolizidine alkaloids usually consist of a base portion (necine) which is a 1-hydroxymethylpyrrolizidine derivative. A second hydroxy group is often present at C-7 and the most common necine is retronecine (1). The necines differ in the degree of hydroxylation, stereochemistry of substituents, and degree of unsaturation of the rings. Pyrrolizidine alkaloids usually occur as monoesters, diesters, or cyclic diesters. The ring sizes are generally 11-membered as in monocrotaline (2) or 12-membered as in retrorsine (3). The esterifying



(1)



(2)



(3)

portions (necic acids) have unusual structures. They contain from five to ten carbon atoms and differ in the degree of chain branching, hydroxylation, and unsaturation. Most necic acids are dicarboxylic and contain ten carbon atoms.

Pyrrolizidine alkaloids are important because of the wide range of biological activities which they exhibit. Alkaloids such as monocrotaline (2) or retrorsine (3) which contain a 1,2-unsaturated necine are hepatotoxic.[2] Many deaths of livestock have resulted from animals grazing on land containing plants which produce pyrrolizidine alkaloids. These plants are also favourites for many herbal remedies which are known to contribute towards human liver disease. Some species of Lepidoptera do make good use of these alkaloids. The alkaloids are

[1] D. J. Robins, *Nat. Prod. Rep.*, 1984, **1**, 235; 1985, **2**, 213; 1986, **3**, 297; 1987, **4**, 577; 1989, **6**, 221.
[2] A. R. Mattocks, 'Chemistry and Toxicology of Pyrrolizidine Alkaloids,' Academic Press, London, 1986.

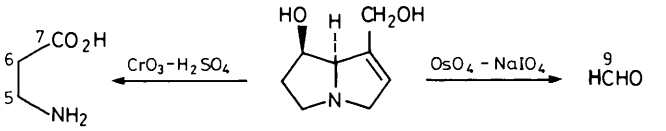
ingested and stored for defensive purposes—the bitter taste makes the butterflies unpalatable to potential predators. In addition, the necine part of the alkaloid can be converted into volatile ketones which act as pheromones for the butterflies.

Early biosynthetic work on pyrrolizidine alkaloids made use of radiotracers (^{3}H, ^{14}C). Labelled alkaloids were isolated from plants, and the location of labels was partially established by the limited number of degradations possible. Nevertheless, the original proposal of Sir Robert Robinson that the 1-hydroxymethylpyrrolizidine system is derived from two molecules of ornithine (4) or putrescine (5) was substantiated.[3] In the same manner, the notion that the ten-carbon necic acids were terpenoid in origin was disproved. They are in fact formed from some of the common α-amino acids. Further progress was slow until it became possible to use precursors labelled with stable isotopes (^{2}H, ^{13}C, ^{15}N), because improvements in n.m.r. spectroscopy allowed the determination of complete labelling patterns. This work required the careful synthesis of precursors selectively labelled with the stable isotope at high enrichment. Much effort was then expended in obtaining high specific incorporations of labelled precursors so that enriched signals could be detected in the n.m.r. spectra of the labelled alkaloids.

2 Biosynthesis of Necines

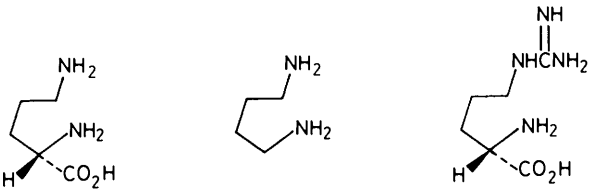
A. Ornithine, Arginine, and Putrescine.—The first experimental evidence concerning the biosynthesis of necines was provided by Nowacki and Byerrum in 1962.[4] They fed [2-^{14}C]ornithine, [1-^{14}C]acetate, and [1-^{14}C]propionate to *Crotalaria spectabilis*, which produces monocrotaline (2). Ornithine was incorporated specifically into the base portion, retronecine (1), whereas the ^{14}C labels from acetate and propionate were found mainly in the necic acid portion. Similar experiments with [2-^{14}C]ornithine and *Senecio* species were also reported by Hughes *et al.*[5] In 1963, Bottomley and Geissman carried out feeding experiments on *S. douglasii*.[6] This species produces a mixture of alkaloids, but they all contain retronecine (1) as the base portion. Total incorporations into the alkaloids (radioactivity in alkaloids ÷ radioactivity in precursor × 100%) of [1,4-^{14}C]putrescine, [2-^{14}C]ornithine, and [5-^{14}C]ornithine were 0.18, 0.30, and 0.75%, respectively. Basic hydrolysis of each alkaloid mixture showed that 94—98% of the radioactivity was located in the base portion. Degradations on retronecine (1) to produce smaller fragments have proved difficult over the years. These workers treated retronecine with osmium tetroxide and sodium periodate. The formaldehyde liberated was trapped as the dimedone derivative which corresponds to C-9 of retronecine (1) (Scheme 1). In all three experiments *ca.* 25% of the retronecine radioactivity was present in this derivative. These results suggest that C-2 and C-5 of ornithine (4) become equivalent in the biosynthetic pathway, probably by formation of putrescine (5).

[3] R. Robinson, 'The Structural Relations of Natural Products,' Clarendon Press, Oxford, 1955.
[4] E. Nowacki and R. U. Byerrum, *Life Sci.*, 1962, **1**, 157.
[5] C. A. Hughes, R. Letcher, and F. L. Warren, *J. Chem. Soc.*, 1964, 4974.
[6] W. Bottomley and T. A. Geissman, *Phytochemistry*, 1964, **3**, 357.



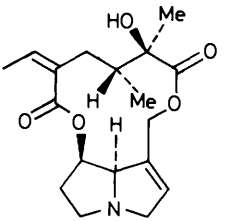
(1)

Scheme 1



(4) (5) (6)

Bale and Crout introduced a most useful improvement in radiotracer feeding experiment strategy by using a double isotope technique to provide a comparison between different feeding experiments with ornithine (4) and arginine (6).[7] When L-[U-^{14}C]arginine and L-[3-^{3}H]arginine were fed in a series of experiments to *Senecio magnificus*, the $^{3}H/^{14}C$ ratio decreased from an initial 4.84 to an average value of 3.0 in senecionine (7). On the other hand, when L-[U-^{14}C]ornithine and L-[3-^{3}H]arginine were employed, the $^{3}H/^{14}C$ ratio fell from 3.62 to 2.2 in senecionine (7). Thus ornithine (4) is a slightly more efficient precursor than arginine (6) for the biosynthesis of retronecine (1). It should be noted that the total incorporations of ^{14}C were low and variable in different feeding experiments using hydroponic techniques, but the measurement of these ratios provides a reliable comparison for the assessment of the efficiency of incorporation of different precursors.



(7)

This strategy was later extended by Robins and Sweeney to measure the relative retention of ^{3}H and ^{14}C on incorporation of D-, L-, and DL-isomers of

[7] N. M. Bale and D. H. G. Crout, *Phytochemistry*, 1975, **14**, 2617.

^{14}C-labelled arginine and ornithine into retrorsine (3) in *Senecio isatideus* using L-[5-^{3}H]arginine as an internal standard.[8] Retronecine (1), the base portion of retrorsine, was shown to be derived entirely from the L-isomers of arginine (6) and ornithine (4).

The involvement of ornithine (4) and/or arginine (6) in the biosynthesis of pyrrolizidine bases *via* putrescine (5) is the subject of some controversy. By using decarboxylase inhibitors, Birecka and co-workers provided evidence that putrescine is largely formed from arginine in *Heliotropium* spp.,[9] whereas in *Senecio* and *Crotalaria* spp., the main route to putrescine is from ornithine.[10] This appears to be at variance with a report by Hartmann *et al.* that putrescine is derived exclusively from arginine in *S. vulgaris*.[11]

When we began our work on the biosynthesis of necines in 1978, we were keen to improve the total incorporations of precursors obtained in feeding experiments, with a view to the subsequent use of stable isotopes in defining the pathways to pyrrolizidine alkaloids. A wide variety of feeding methods were carried out on *Senecio isatideus* which produces retrorsine (3). High total incorporations were obtained by direct adsorption of sterile aqueous solutions of the precursors into xylems of plants growing normally in soil, through stem punctures.[12] A double label technique with various ^{14}C-labelled precursors was used with L-[5-^{3}H]-arginine as a reference in each experiment. Putrescine (4), spermidine (8), and spermine (9), were the most efficient precursors for retrorsine (3) in *S. isatideus*

$$H_2N(CH_2)_3NH(CH_2)_4NHR$$

(8) R = H

(9) R = $(CH_2)_3NH_2$

plants with very satisfactory total incorporations of 1.6—5.2%. Spermidine and spermine are probably utilized *via* putrescine, and these three precursors were incorporated into retrorsine about ten times more efficiently than ornithine (4) or arginine (6). This feeding method has been our favourite together with the wick method when plant stems are sufficiently strong. Degradations were carried out on the samples of labelled retronecine obtained by basic hydrolysis of retrorsine. As before, formaldehyde was obtained by oxidative cleavage of retronecine to give C-9. In addition, modified Kuhn–Roth oxidation of retronecine gave β-alanine isolated as its 2,4-dinitrophenyl derivative, corresponding to C-(5 + 6 + 7) of retronecine (Scheme 1). In experiments with [5-^{14}C]ornithine, [1,4-^{14}C]-putrescine, spermidine, and spermine (both labelled [1,4-^{14}C] in the tetramethylene portion), *ca.* 25% of the retronecine radioactivity was found both in C-9 and in C-(5 + 6 + 7). These results support the view that retronecine (1) is

[8] D. J. Robins and J. R. Sweeney, *Phytochemistry*, 1983, **22**, 457.
[9] H. Birecka, M. Birecki, and M. W. Frohlich, *Plant Physiol.*, 1987, **84**, 42.
[10] H. Birecka, M. Birecki, E. J. Cohen, A. J. Bitonti, and P. P. McCann, *Plant Physiol.*, 1988, **86**, 224.
[11] T. Hartmann, H. Sander, R. Adolph, and G. Toppel, *Planta*, 1988, **175**, 82.
[12] D. J. Robins and J. R. Sweeney, *J. Chem. Soc., Chem. Commun.*, 1979, 120; *J. Chem. Soc., Perkin Trans. 1*, 1981, 3083.

derived from two molecules of putrescine (5) formed from ornithine (4) or arginine (6).[12]

At this point in the biosynthetic studies it was clear that further progress was hampered by the lack of good degradations of retronecine (1) which would provide detailed labelling patterns in alkaloids after feeding experiments with radiolabelled precursors. However, the substantially improved total incorporations obtained[12] suggested that attempts should be made to use ^{13}C-labelled precursors in order to obtain complete labelling patterns in alkaloids by ^{13}C n.m.r. spectroscopy. Accordingly, the ^{13}C natural abundance n.m.r. spectrum of retronecine hydrochloride was assigned by using off resonance and single frequency decoupled spectra (Figure 1a). These assignments were made before starting the biosynthetic work.[13] Then [1,4-$^{13}C_2$]putrescine dihydrochloride (10) was made as shown in Scheme 2, and fed to young *Senecio isatideus* plants. It was important to use small plants to avoid excessive dilution by endogenous unlabelled alkaloid. In all stable isotope work it is desirable to feed ^{14}C-labelled precursor with the ^{13}C-labelled material to provide an independent check on the incorporation. Reasonable specific incorporations per C_4 unit (molar activity of product $\times \frac{1}{2} \div$ molar activity of precursor $\times$ 100%) into retrorsine were observed with a mixture of [1,4-$^{13}C_2$]putrescine and [1,4-^{14}C]putrescine. Comparison of the $^{13}C\{^1H\}$ n.m.r. spectra for the ^{13}C-labelled retronecine hydrochloride did show enhanced signals for C-3, C-5, C-8, and C-9, but considerable broadening of the signals was observed due *e.g.* to ^{13}C–N–^{13}C from C-5 to C-8; or geminal coupling C-3 to C-5 or C-8, and C-9 to C-8 arising from combination of two molecules of ^{13}C-labelled putrescine in the formation of retronecine.[13] In order to reduce these extra couplings, [1-^{13}C]putrescine dihydrochloride (11) was prepared as outlined in Scheme 3. The best feeding experiments with this precursor involved using large numbers (102!) of young *S. isatideus* plants. A sample of retronecine hydrochloride was produced which showed four nearly equally enriched carbon atoms corresponding to C-3, C-5, C-8, and C-9 of retronecine (1) (Figure 1b).[13]

In a number of our early experiments with ^{13}C-labelled putrescines, ^{13}C enrichments were low, due mainly to dilution of the labelled alkaloid with appreciable quantities of endogenous unlabelled alkaloid. A way round this problem is to use ^{13}C–^{13}C doubly labelled precursors, because smaller incorporations of ^{13}C can be detected by the presence of ^{13}C–^{13}C doublets straddling the natural abundance signals in the ^{13}C n.m.r. spectrum. Therefore [2,3-$^{13}C_2$]-putrescine dihydrochloride was made as shown in Scheme 2 except that [1,2-$^{13}C_2$]-1,2-dibromoethane was treated with unlabelled potassium cyanide. The doubly ^{13}C-labelled putrescine was fed to *S. isatideus* and the labelled retronecine hydrochloride showed a pair of doublets corresponding to C-1 and C-2 (J 71 Hz), and another pair due to C-6 and C-7 (J 34 Hz) (Figure 1c). Enrichments at all four labelled sites were approximately equal.[13]

[13] H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 146; *J. Chem. Soc., Perkin Trans. 1*, 1985, 101.

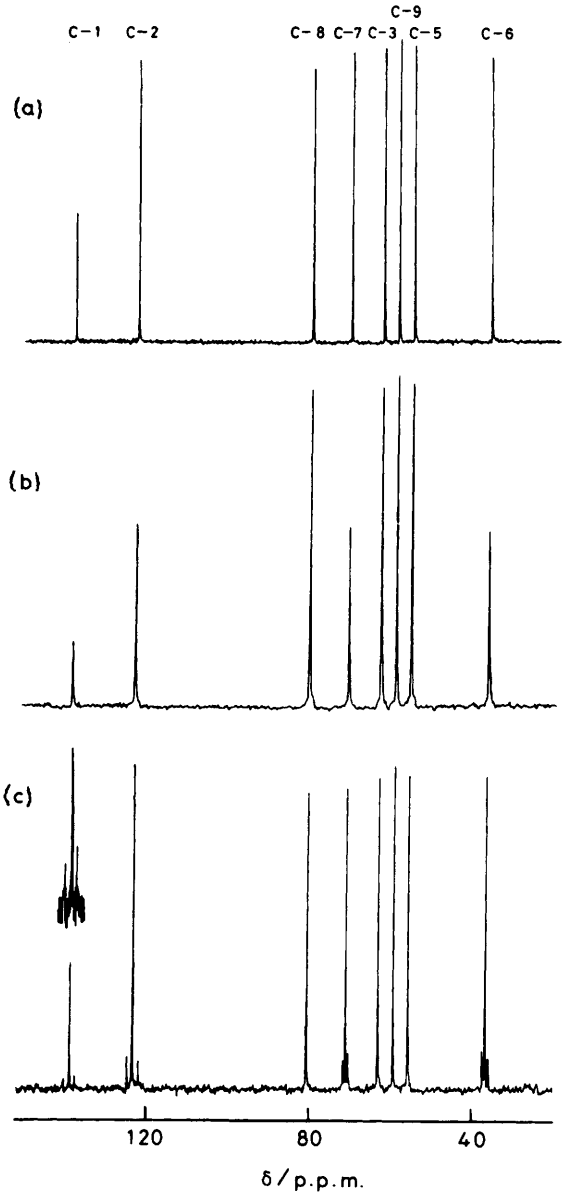
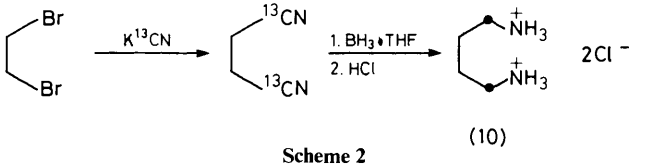
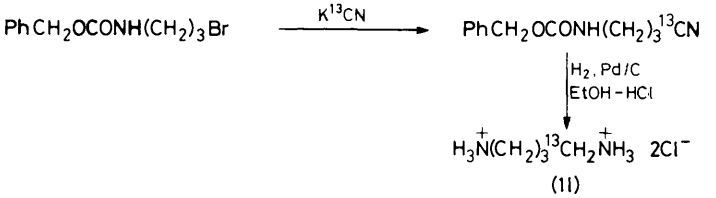


Figure 1 *Proton-noise decoupled* ^{13}C *n.m.r. spectra of retronecine* (1) *hydrochloride in deuterium oxide: (a) unlabelled material; (b) enriched with* [1-$^{13}C_1$]*putrescine dihydrochloride; (c) enriched with* [2,3-$^{13}C_2$]*putrescine dihydrochloride*



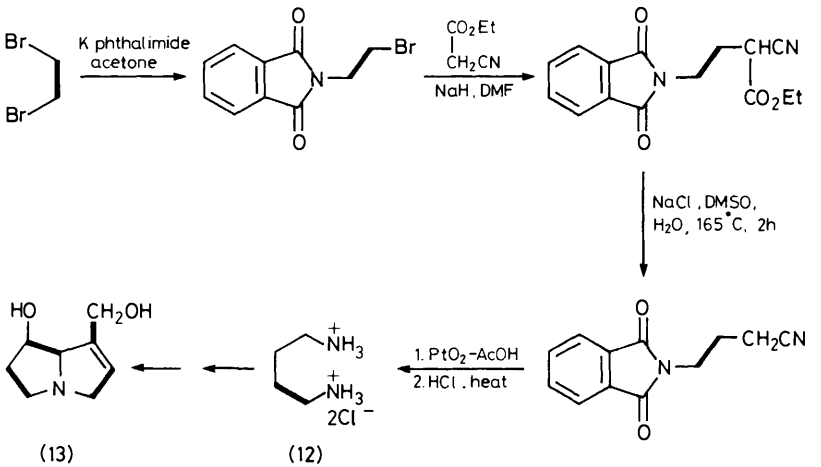
(10)

Scheme 2



(11)

Scheme 3



(13) (12)

Scheme 4

The use of [2,3-$^{13}C_2$]putrescine dihydrochloride had resulted in a sample of retronecine which showed couplings for half of the carbon atoms. The best precursor to study putrescine metabolism is [1,2-$^{13}C_2$]putrescine, which was made as outlined in Scheme 4.[14] The source of the double label was again [1,2-$^{13}C_2$]-1,2-dibromoethane. This was converted into a phthalimide derivative and the extra two carbon atoms required were provided by reaction with ethyl cyanoacetate. Removal of the ester group, hydrogenation of the nitrile and hydrolysis of the protecting group afforded [1,2-$^{13}C_2$]putrescine dihydrochloride (12) in 19% overall yield. When this was fed to *S. isatideus*, retronecine (13)

[14] D. J. Robins, *J. Chem. Res.*, (*S*), 1983, 326.

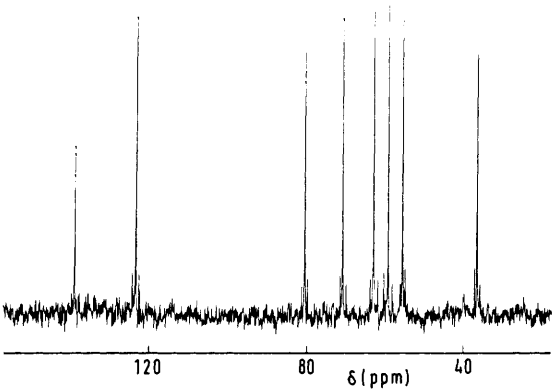


Figure 2 25 MHz ^{13}C-{^{1}H} *N.m.r. spectrum of retronecine* (13) *hydrochloride* (14 mg) *in* D_2O *derived from* [1,2-$^{13}C_2$]*putrescine dihydrochloride* (12)

hydrochloride was obtained which displayed four pairs of doublets, *i.e.* all eight carbon atoms were enriched with ^{13}C. The distinctive labelling pattern shown in Figure 2 is reminiscent of those obtained for polyketides using [1,2-$^{13}C_2$]acetate. [1,2-$^{13}C_2$]Putrescine dihydrochloride (12) is clearly the preferred precursor for biosynthetic studies on metabolites derived from putrescine.

B. Homospermidine.—The complete labelling patterns obtained in retronecine (1) after feeding different ^{13}C-labelled putrescines all lead to the conclusion that two molecules of putrescine (5) are incorporated to about the same extent into retronecine. This suggests, but provides no evidence, that a later C_4–N–C_4 symmetrical intermediate is involved in the biosynthetic pathway. We believed that the use of a ^{13}C–^{15}N doubly labelled precursor would furnish this evidence. Therefore [^{13}C–^{15}N]putrescine dihydrochloride (14) was prepared as outlined in Scheme 5 using potassium [^{13}C–^{15}N] cyanide as the source of the labels.[15] After the feeding experiment to *S. isatideus* had been carried out, the sample of retronecine hydrochloride produced showed four enriched carbon atoms in the ^{13}C n.m.r. spectrum corresponding to C-3, C-5, C-8, and C-9—an exactly similar pattern to that obtained with [1-^{13}C]putrescine dihydrochloride. In addition, the resolution-enhanced spectrum showed the presence of doublets around the signals for C-3 and C-5 with about equal enrichments (Figure 3). The fact that the sample of retronecine is composed of equal amounts of the labelled species (15) provides compelling evidence that a later C_4–N–C_4 symmetrical intermediate is involved in retronecine biosynthesis. Grue–Sorensen and Spenser carried out analogous experiments on *Senecio vulgaris*, and degraded a mixture of alkaloids to produce their labelled retronecine, which showed a similar labelling pattern.[16]

An interesting contrast in the biosynthetic pathways is provided by lupinine (18). It is not surprising that lupinine was shown to be formed from two

[15] H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 554.
[16] G. Grue-Sorensen and I. D. Spenser, *J. Am. Chem. Soc.*, 1981, **103**, 3208; *Can. J. Chem.*, 1982, **60**, 643.

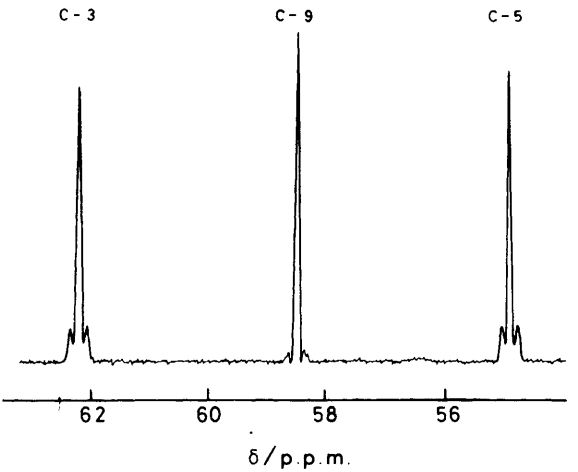
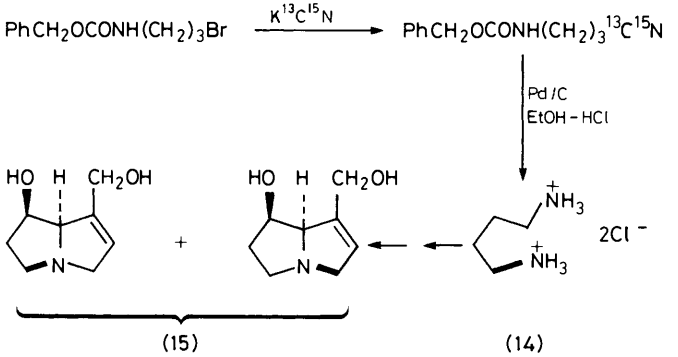


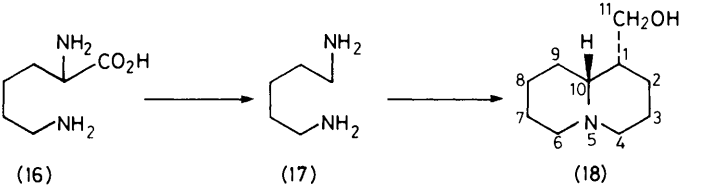
Figure 3 *Part of the resolution enhanced proton-noise decoupled* ^{13}C *n.m.r. spectrum of retronecine* (15) *hydrochloride enriched with* [1-*amino*-^{15}N,1-^{13}C]*putrescine dihydrochloride* (14)



Scheme 5

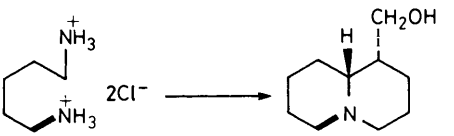
molecules of lysine (16)[17] *via* cadaverine (17),[18] using ^{13}C–^{13}C doubly labelled precursors (Scheme 6). However, when [^{13}C–^{15}N]cadaverine dihydrochloride (19) was prepared and fed to *Lupinus luteus*, the lupinine (20) obtained showed only one ^{13}C–^{15}N bond in the $^{13}C\{^1H\}$ n.m.r. spectrum associated with C-6 of lupinine (Scheme 7).[19,20] Therefore, it is clear that no later symmetrical

[17] J. Rana and D. J. Robins, *J. Chem. Res.*, (*S*), 1984, 164.
[18] D. J. Robins and G. N. Sheldrake, *J. Chem. Res.*, (*S*), 1987, 256; (*M*), 1987, 2101.
[19] J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1984, 81; *J. Chem. Soc., Perkin Trans. 1*, 1986, 1133.
[20] W. M. Golebiewski and I. D. Spenser, *J. Chem. Soc., Chem. Commun.*, 1983, 1509; *J. Am. Chem. Soc.*, 1984, **106**, 7925.



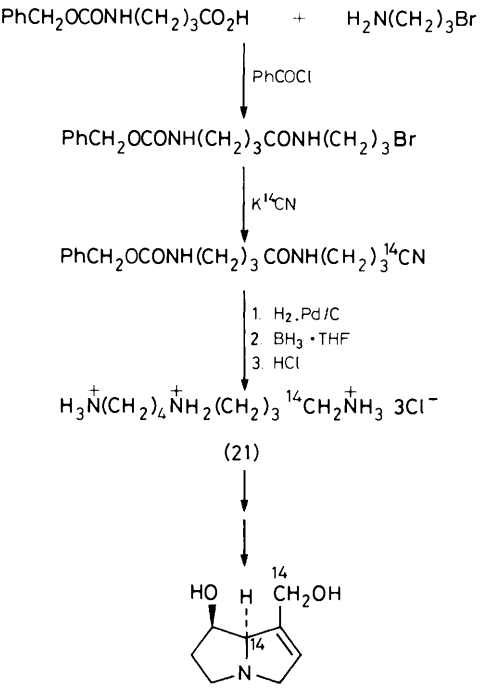
(16) (17) (18)

Scheme 6

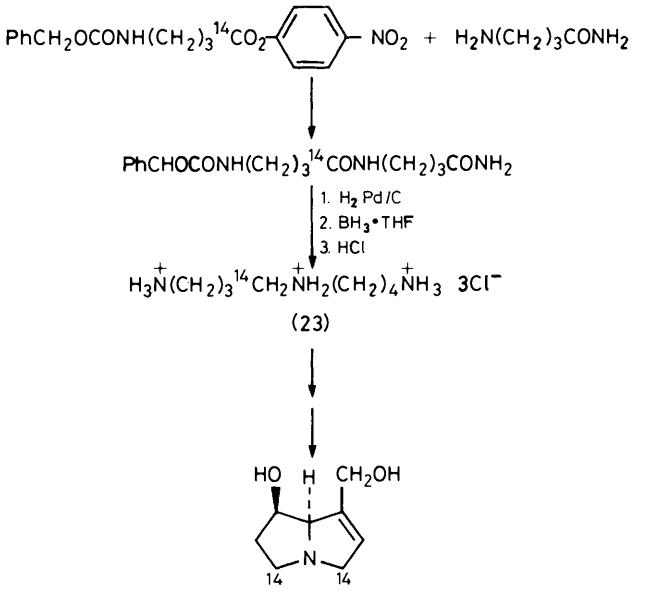


(19) (20)

Scheme 7



Scheme 8



Scheme 9

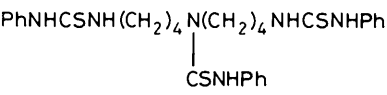
intermediate of the type C_5–N–C_5 is involved in lupinine biosynthesis. This finding provides another caution against taking anything for granted in biosynthetic work, even when close structural analogies are involved.

It was necessary to identify the later symmetrical C_4–N–C_4 intermediate in retronecine biosynthesis. We felt that homospermidine was the best candidate, as it is a known plant constituent, particularly in sandalwood.[21] [1,9-^{14}C]Homospermidine trihydrochloride (21) was prepared as shown in Scheme 8.[15] When this material was fed to *Senecio isatideus*, a reasonable total incorporation of 0.5% into retrorsine was obtained. Hydrolysis of the alkaloid and degradation of the retronecine established that 44% of the radioactivity was present at C-9 and 2% was in C-(5 + 6 + 7). These results are consistent with the likely labelling pattern (22). A complementary labelling pattern was obtained after synthesizing [4,6-^{14}C]homospermidine trihydrochloride (23) as outlined in Scheme 9.[22] A comparable total incorporation of 0.7% was achieved in retrorsine, and degradation showed that 3% of the radioactivity was at C-9 and 46% was located in C-(5 + 6 + 7), suggesting the labelling pattern (24) in retronecine.

In order to provide evidence for the presence of homospermidine in *S. isatideus* plants, an intermediate trapping experiment was carried out.[22] DL-[5-^{14}C]-

[21] R. Kuttan, A. N. Radhakrishnan, T. Spande, and B. Witkop, *Biochemistry*, 1971, **10**, 361.
[22] H. A. Khan and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1985, 819.

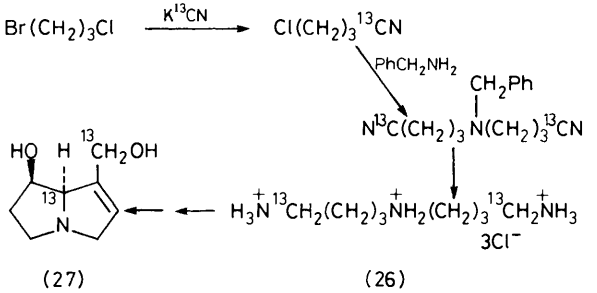
Ornithine was fed to one plant, and after one day, the plant was harvested and inactive homospermidine trihydrochloride was added to the acidic extract. The *N*-phenylamino(thiocarbonyl) derivative (25) of homospermidine was formed by



(25)

addition of isothiocyanatobenzene to the basified extract. T.l.c. of the isolated derivatives showed that most of the radioactivity (80%) was due to the derivative of putrescine, but *ca.* 10% was located with the homospermidine derivative. This derivative was recrystallized to constant specific radioactivity corresponding to about 0.5% of the radioactivity orginally fed. Homospermidine is therefore formed from ornithine in *S. isatideus.*

The use of ^{14}C-labelled homospermidines had resulted in incomplete labelling patterns for the retronecine samples. Accordingly, it was decided to prepare [1,9-$^{13}C_2$]homospermidine trihydrochloride (26). It was essential to introduce two ^{13}C atoms into each homospermidine molecule and so a different synthetic route was employed (Scheme 10) based on work of Bergeron *et al.*[23] This material was



Scheme 10

fed to *S. isatideus* and the sample of retronecine (27) hydrochloride obtained showed the presence of two doublets of equal enrichment around the natural abundance signals for C-8 and C-9 in the resolution-enhanced $^{13}C\{^1H\}$ n.m.r. spectrum (Figure 4). The observation of a geminal coupling constant of *ca.* 6 Hz between C-8 and C-9 of retronecine (27) is convincing evidence for the intact incorporation of homospermidine into retronecine.[24] Homospermidine has been detected in *Heliotropium indicum* by Birecka and co-workers.[25]

While visiting the Royal Botanic Garden, Edinburgh, in 1984, the splendid climbing plant *Senecio pleistocephalus* was spotted in a greenhouse. Cuttings

[23] R. J. Bergeron, P. S. Burton, K. A. McGovern, and S. J. Kline, *Synthesis*, 1981, 732.
[24] J. Rana and D. J. Robins, *J. Chem. Res.*, (*S*), 1983, 146.
[25] H. Birecka, T. E. DiNolfo, W. B. Martin, and M. W. Frohlich, *Phytochemistry*, 1984, **23**, 991.

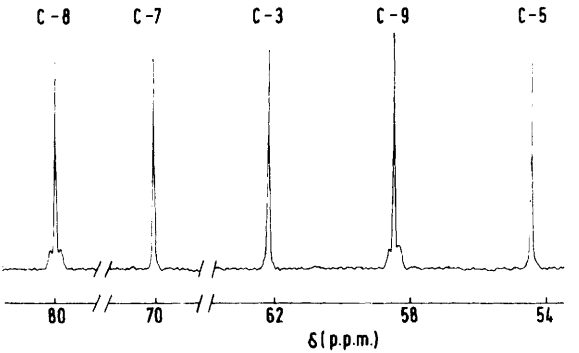
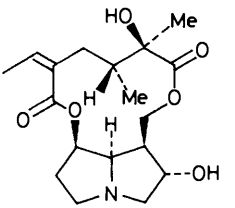


Figure 4 25.16 MHz ^{13}C-$\{^1H\}$ *N.m.r. spectrum of retronecine* (27) *hydrochloride* (20 mg) *in* D_2O *derived from* [1,9-$^{13}C_2$]*homospermidine trihydrochloride* (26)

were acquired and the plants grown on to establish that they produce rosmarinine (28) as the sole alkaloid constituent. This enabled biosynthetic



(28)

experiments to be carried out on a different base, rosmarinecine. Initial experiments were carried out with ^{13}C-labelled putrescines. We were agreeably surprised to obtain a specific incorporation of 22% per C_4 unit into rosmarinine with [1-^{13}C]putrescine dihydrochloride on young plants (Figure 5a).[26] The enriched signals as expected were associated with C-3, C-5, C-8, and C-9. Use of [2,3-$^{13}C_2$]putrescine dihydrochloride also produced a sample of rosmarinine with good enrichment shown by doublets around the natural abundance signals for C-1, C-2, C-6, and C-7 (Figure 5b). Feeding of [^{13}C–^{15}N]putrescine dihydrochloride probably gave rise to doublets around C-3 and C-5 of rosmarinine (Figure 6) but here the situation is less clear because part of each doublet is obscured by the natural abundance signals. When [1,9-$^{13}C_2$]homospermidine trihydrochloride (26) was fed to *S. pleistocephalus*, two enriched signals were observed in the $^{13}C\{^1H\}$ n.m.r. spectrum of rosmarinine (Figure 7), *i.e.* the geminal coupling constant between C-8 and C-9 in rosmarinine is zero. Nevertheless, it can be concluded that rosmarinecine, like retronecine is formed from two molecules of putrescine *via* homospermidine.[26]

[26] H. A. Kelly and D. J. Robins, *J. Chem. Soc., Perkin Trans 1*, 1987, 177.

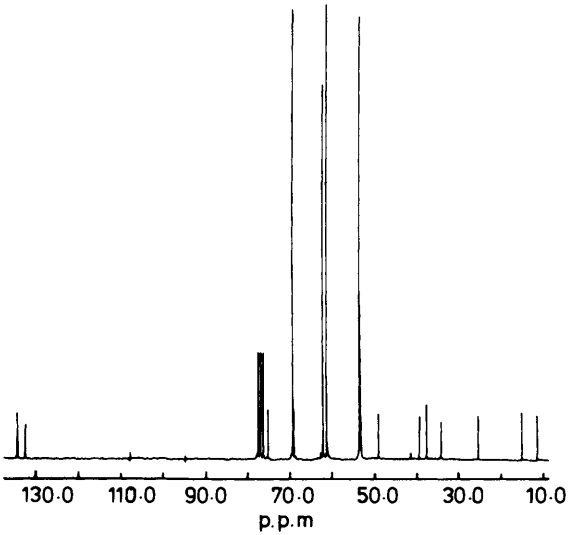


Figure 5a 50 MHz ^{13}C-$\{^1H\}$ *N.m.r. spectrum of rosmarinine* (28) *in* $CDCl_3$ *enriched with* [1-^{13}C]*putrescine dihydrochloride* (11)

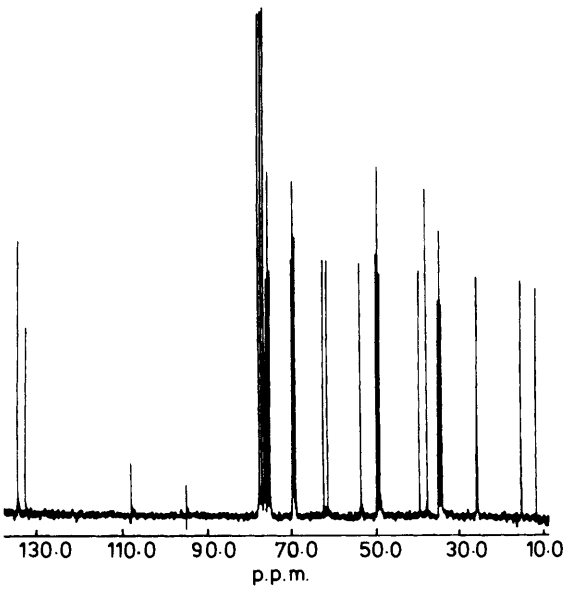


Figure 5b 50 MHz ^{13}C-$\{^1H\}$ *N.m.r. spectrum of rosmarinine* (28) *in* $CDCl_3$ *enriched with* [2,3-$^{13}C_2$]*putrescine dihydrochloride*

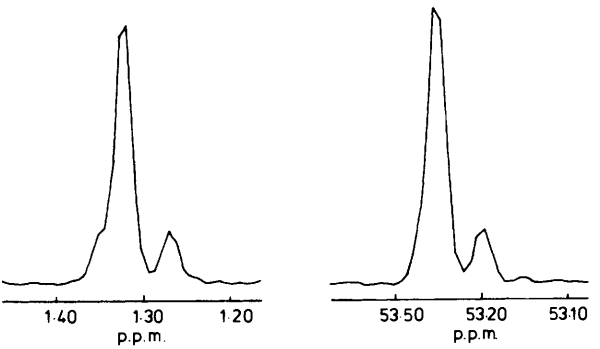


Figure 6 *Part of the* 50 MHz *resolution-enhanced* ^{13}C-$\{^1H\}$ *n.m.r. spectrum of rosmarinine* (28) *in* $CDCl_3$ *enriched with* [1-*amino*-^{15}N,1-^{13}C]*putrescine dihydrochloride* (14)

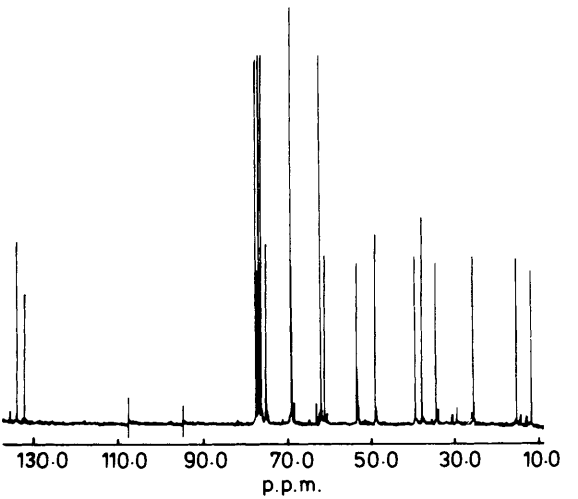
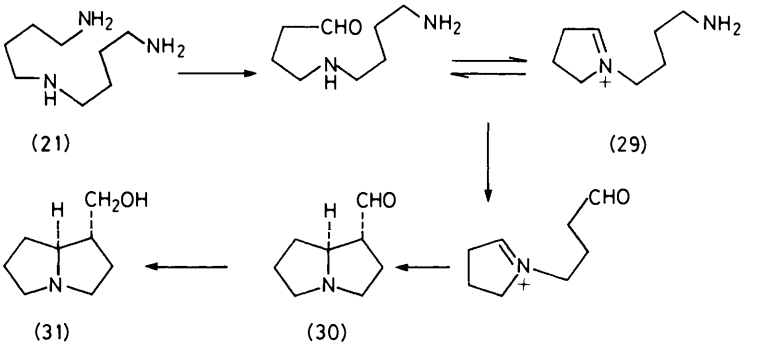
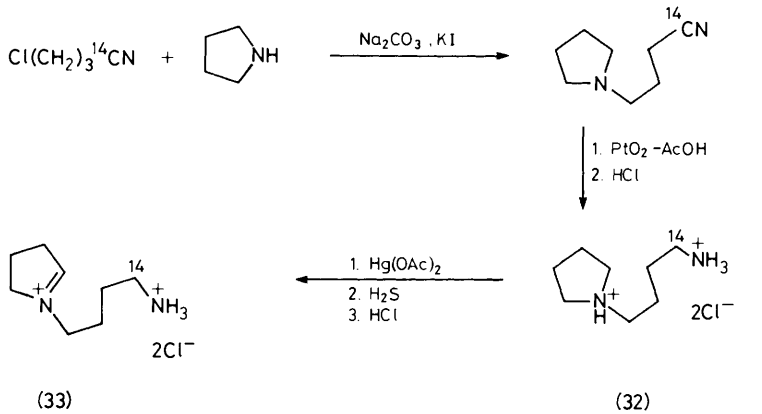


Figure 7 50 MHz ^{13}C-$\{^1H\}$ *N.m.r. spectrum of rosmarinine* (28) *in* $CDCl_3$ *enriched with* [1,9-$^{13}C_2$]*homospermidine trihydrochloride* (26)

Clues to the identity of later intermediates in the biosynthetic pathways to necines came from consideration of the likely metabolism of homospermidine (21). I felt that diamine oxidases are probably involved. Oxidation of one primary amino group in homospermidine by a diamine oxidase (or transaminase) would give an aldehyde in equilibrium with the iminium ion (29). Oxidation of the remaining primary amino group would afford an aldehyde which could undergo cyclization to 1-formylpyrrolizidine (30). A reduction would then yield 1-hydroxymethylpyrrolizidine (31), a known necine (Scheme 11). I decided to test this theory by treating homospermidine with the diamine oxidase isolated from pea seedlings. This was left for a week to allow the oxidations to take place,



Scheme 11



Scheme 12

followed by a non-enzymic cyclization, then either chemical reduction or use of a coupled dehydrogenase afforded trachelanthamidine (31) in 40 and 27% yields, respectively.[27] No optical activity could be detected in the base (31), but this point deserves further investigation. The ready formation of trachelanthamidine (31) under physiological conditions from homospermidine using readily available enzymes did indicate that such reactions are involved in the biosynthesis of necines, and further suggested worthwhile candidates for assessment as intermediates in the pathway—iminium ion (29) and 1-hydroxymethylpyrrolizidine (31).

C. Iminium Ions.—Consideration of the enzymic conversion of homospermidine (21) into trachelanthamidine (31) suggested iminium ion (29) as a possible intermediate in the biosynthetic pathway. The ^{14}C-labelled iminium ion (33) was

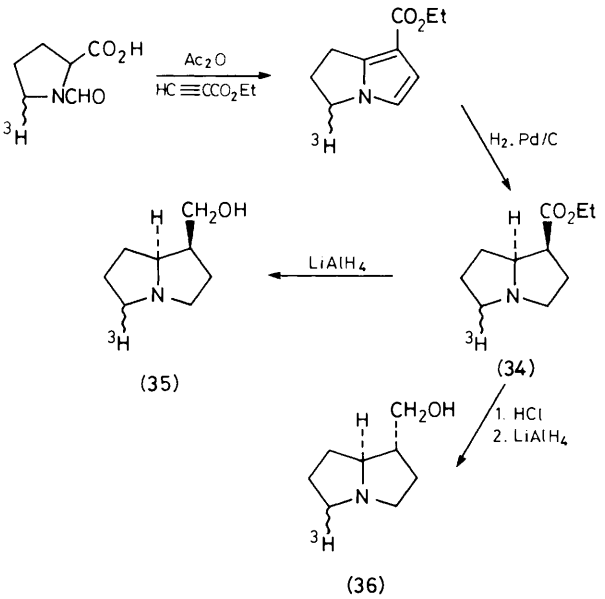
[27] D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1982, 1289.

prepared as shown in Scheme 12.[28] The key step in this route was the oxidation of the saturated salt (32) with mercuric acetate. Although literature precedent indicated that formation of an endocyclic double bond should take place, it was felt desirable to confirm the location of the double bond. A sample of unlabelled iminium salt was reduced with sodium cyanoborodeuteride to yield a mono-deuteriated product. A ^{1}H n.m.r. spectrum of this material showed a three-proton multiplet at δ 2.95, whereas the ^{1}H n.m.r. spectrum of the saturated salt contained a four-proton multiplet at this chemical shift. The ^{14}C-labelled iminium ion was fed together with [1,4-^{3}H]putrescine (^{3}H/^{14}C ratio 12.3) to *Senecio isatideus*. Retrosine (3) was isolated with a ^{14}C specific incorporation of 4.5% and a ^{3}H/^{14}C ratio of 9.8. The iminium salt was also fed in a similar double-label experiment to *S. pleistocephalus* and rosmarinine (28) was isolated with a ^{14}C specific incorporation of 6.5% and ^{3}H/^{14}C ratio reduced from 5 to 2.9. (Similar ratios and specific incorporations were observed in each base after hydrolysis.) The iminium salt is therefore an efficient precursor for retronecine (1) and rosmarinecine, and, from the decrease in ^{3}H/^{14}C ratios, it is incorporated more efficiently into these necines than is putrescine.

Furthermore, evidence for the presence of the iminium ion (29) in *Senecio* plants was obtained by an intermediate trapping experiment.[28] [1,4-^{14}C]Putrescine dihydrochloride was fed to one *S. pleistocephalus* plant, and after one day the plant was harvested and inactive iminium ion (29) was added to the methanolic extract, followed by sodium borohydride. The reduced products were derivatized with isothiocyanatobenzene. The purified derivative of *N*-(4-aminobutyl)pyrrolidine contained 0.4% of the original radioactivity. It should be noted that the saturated salt (32) is also a reasonably good precursor for both retronecine and rosmarinecine, but an intermediate trapping experiment gave a derivative with very low radioactivity. Thus it is clear that in two *Senecio* species the saturated salt (32) can be oxidized to the iminium ion (33), but the iminium ion is the likely biosynthetic intermediate.

D. 1-Hydroxymethylpyrrolizidines.—The second set of intermediates suggested by the enzymic conversion of homospermidine (21) into trachelanthamidine (31) comprises trachelanthamidine itself and its stereoisomers. For the preparation of ^{3}H-labelled material the synthesis of Pizzorno and Albonico[29] was adapted. 1,3-Dipolar cycloaddition of *N*-formyl-[5-^{3}H]-L-proline with ethyl propiolate gave a dihydropyrrolizine ester (Scheme 13). Stereospecific *cis*-hydrogenation of this pyrrole gave the *endo*-ester (34) which was reduced to afford (±)-[5-^{3}H]-isoretronecanol (35). Epimerization of the *endo*-ester was achieved using base or (better) acid, then reduction gave (±)-[5-^{3}H]trachelanthamidine (36).[30] Careful analysis of the ^{1}H and ^{13}C n.m.r. spectra of both ^{3}H-labelled necines showed that each racemate contained less than 3% of the other. These ^{3}H-labelled racemates were fed together with ^{14}C-labelled putrescine (^{3}H/^{14}C ratio 10) to *Senecio*

[28] H. A. Kelly and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1988, 329.
[29] M. T. Pizzorno and S. M. Albonico, *J. Org. Chem.*, 1974, **39**, 731.
[30] E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1986, 250.



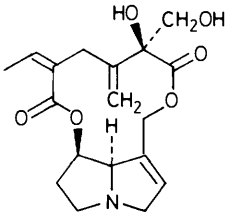
Scheme 13

isatideus and *S. pleistocephalus*. The ^3H specific incorporation into rosmarinine (28) was 2.4% with a $^3H/^{14}C$ ratio of 17 after feeding isoretronecanol (35) and $<0.1\%$ with a $^3H/^{14}C$ ratio of <0.5 after feeding trachelanthamidine (36). Isoretronecanol is thus incorporated 34 times more efficiently into rosmarinine than is trachelanthamidine. Also isoretronecanol is a more efficient precursor for rosmarinine than putrescine, particularly if it is assumed that only one enantiomer is used in the biosynthesis. A contrasting result was obtained with *S. isatideus*.[30] After feeding isoretronecanol (35) the ^3H specific incorporation was 0.3% with a $^3H/^{14}C$ ratio of 0.7, and with trachelanthamidine (36) it was 2.8% with a $^3H/^{14}C$ ratio of 14.3. Trachelanthamidine is therefore a much better precursor for retrorsine (3) than isoretronecanol, and trachelanthamidine is incorporated more efficiently into retrorsine than putrescine. Basic hydrolysis of the labelled alkaloids confirmed that the radioactivity was confined to the base portions, and most of this activity was present in β-alanine obtained by chromic acid oxidation of the necines.

Leete and Rana reported similar results for the incorporation of ^3H-labelled trachelanthamidine rather than isoretronecanol into riddelliine (37) (base portion is retronecine) in *Senecio riddellii*.[31]

Evidence supporting 1-hydroxymethylpyrrolizidines as biosynthetic inter-

[31] J. Rana and E. Leete, *J. Chem. Soc., Chem. Commun.*, 1985, 1742; E. Leete and J. Rana, *J. Nat. Prod.*, 1986, **49**, 838.



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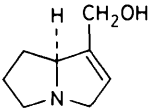

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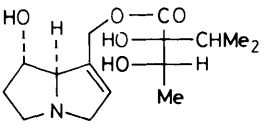


(39)

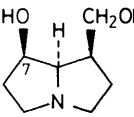
mediates was provided by a different type of experiment. Pulsed labelling experiments using $^{14}CO_2$ on *Heliotropium spathulatum* were carried out by Birecka and Catalfamo.[32] The plants produce trachelanthamidine (31), supinidine (38), and retronecine (1). After the plants had been exposed to $^{14}CO_2$ for different lengths of time, the changes in specific activities of the three bases were consistent with the sequence: (31)→(38)→(1). Pulsed feeding of $^{14}CO_2$ has also been used to produce pyrrolizidine alkaloid mixtures with high specific activities from *Senecio vulgaris*.[33]

The data were extended recently by feeding ^{3}H-labelled 1-hydroxymethylpyrrolizidines with [1,4-^{14}C]putrescine to *Cynoglossum officinale* (Boraginaceae) which produces echinatine (39), containing heliotridine as base portion.[34] Trachelanthamidine (36) was a reasonable precursor for echinatine (^{3}H specific incorporation 0.35%; $^{3}H/^{14}C$ ratio increased from 10 to 17) but isoretronecanol (35) was poorly incorporated into echinatine (^{3}H specific incorporation 0.04%; $^{3}H/^{14}C$ ratio decreased from 10 to 1.0). (Incorporations into plants of the Boraginaceae family have generally been an order of magnitude lower than those obtained with *Senecio* spp.)

Our results from feeding ^{3}H-labelled 1-hydroxymethylpyrrolizidines show that isoretronecanol (35) is a good precursor for rosmarinecine, and trachelanthamidine (36) is incorporated efficiently into retronecine (1) and heliotridine. Epimerization of either trachelanthamidine or isoretronecanol does not appear to take place during the biosynthesis of the alkaloids. Thus the two pathways probably diverge prior to the formation of the alcohols during the cyclization of the iminium ion. Alternatively, epimerization could occur at the aldehyde (30) stage.

The final stages in the biosynthetic pathways to a number of necines are now under scrutiny. Two hydroxylations are required from isoretronecanol to form rosmarinecine. Hydroxylation at C-7 may occur first, because ^{3}H-labelled platynecine (40) was incorporated efficiently into rosmarinine (28) in *Senecio pleistocephalus* plants.[35] Conversion of trachelanthamidine (31) into retronecine (1) may involve two hydroxylation processes (at C-2 and C-7), followed by loss of the elements of water.

[32] H. Birecka and J. L. Catalfamo, *Phytochemistry*, 1982, **21**, 2645.
[33] H. J. Segall, C. H. Brown, and D. F. Paige, *J. Labelled Compd. Radiopharm.*, 1983, **20**, 671.
[34] E. K. Kunec and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1989, 1437.
[35] H. A. Kelly and D. J. Robins, unpublished results.



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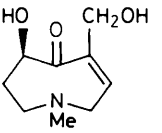
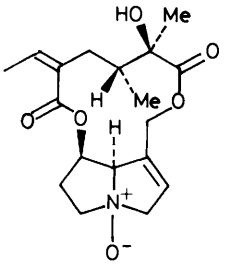


(41)

E. Otonecine.—About 30 pyrrolizidine alkaloids contain otonecine (41) as the base portion. No biosynthetic studies have been reported on otonecine, although Hartmann and co-workers demonstrated that levels of senecionine *N*-oxide (42) decreased with time while amounts of senkirkine (43) increased in root cultures of *Senecio vernalis*.[36]



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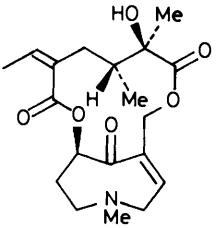

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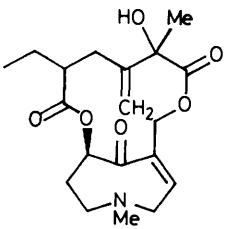
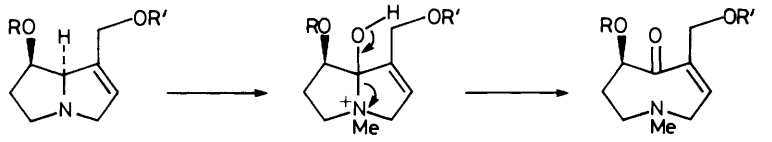


(44)

We tried in vain for a number of years to study the biosynthesis of otonecine, but plants from *Doronicum* and *Ligularia* spp. (Compositae) gave incorporations into alkaloids that were too low for reasonable biosynthetic work. Eventually, we obtained *Emilia flammea* (Compositae) and isolated emiline, which led to a revision of structure to the 12-membered alkaloid (44).[37] Experiments with radioisotopes on *E. flammea* demonstrated that otonecine is formed by the expected biosynthetic pathway. Putrescine (5), homospermidine (21), the iminium ion (33), trachelanthamidine (36), and retronecine (1) were all good precursors.[38] The incorporation of intact pyrrolizidine nuclei in precursors (36) and (1) established that the C(4)–N(8) bond is broken at a late stage in the biosynthetic pathway, probably by hydroxylation at C-8 of retronecine and *N*-methylation, followed by ring cleavage (Scheme 14).

As a result of the extensive studies on the biosynthesis of necines using precursors labelled with radioisotopes and stable isotopes, the main intermediates in the pathways to most types of necine have been established. These are shown in Scheme 15 with known intermediates placed in rectangular boxes. Decarboxylation of L-ornithine (4) yields putrescine (5). Alternatively, putrescine can be produced by decarboxylation of L-arginine (6) and removal of the guanido

[36] G. Toppel, L. Witte, B. Riebesehl, K. v. Borstel, and T. Hartmann, *Plant Cell Rep.*, 1987, **6**, 466.
[37] R. H. Barbour and D. J. Robins, *Phytochemistry*, 1987, **26**, 2430.
[38] H. A. Kelly, E. K. Kunec, M. Rodgers, and D. J. Robins, *J. Chem. Res.*, (*S*), 1989, in press.



Scheme 14

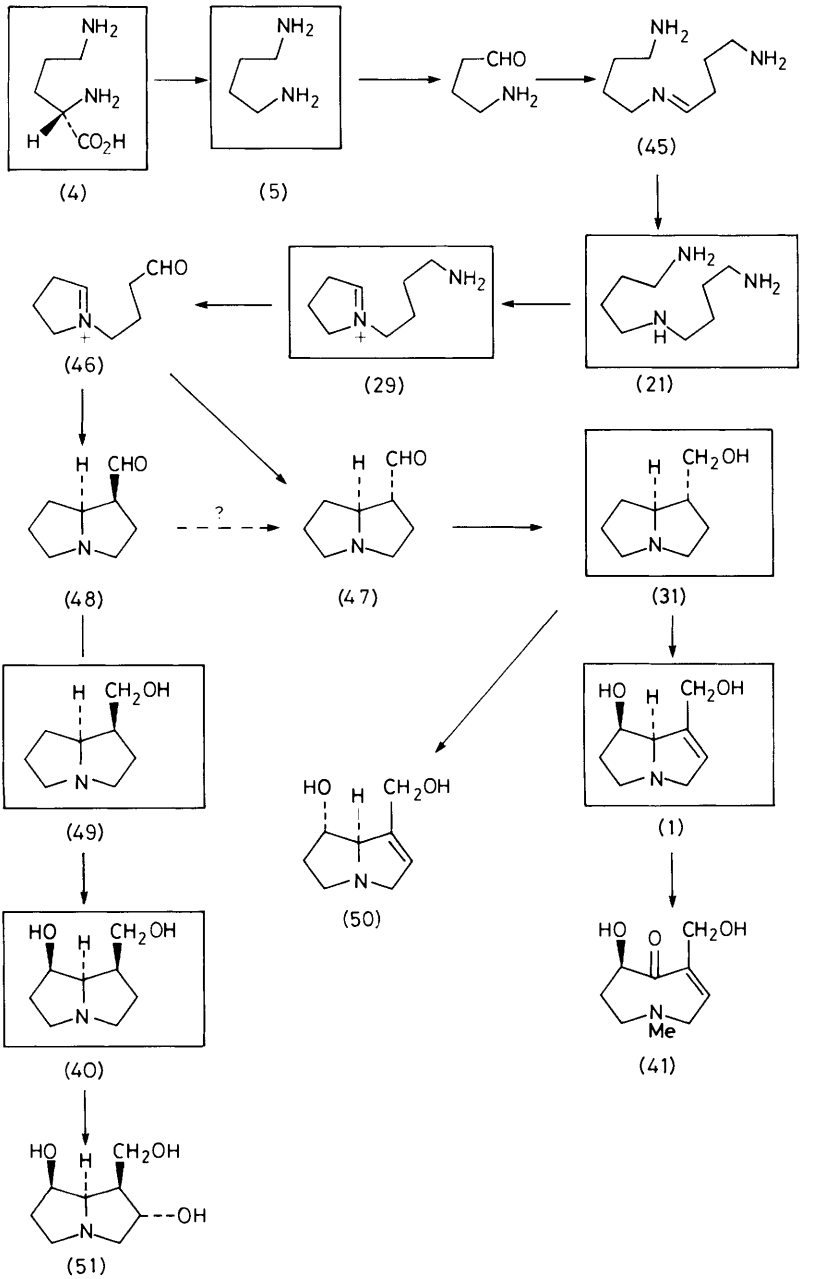
residue. Oxidation of putrescine gives 4-aminobutanal, which can couple with another molecule of putrescine to afford an imine (45) which gives homospermidine (21) on reduction. Oxidation of one of the primary amino groups of homospermidine leads to the iminium ion (29) which undergoes oxidation at the remaining primary amino group to yield aldehyde (46). This aldehyde may cyclize in two ways to give either trachelanthamidine (31) by reduction of the aldehyde (47) or isoretronecanol (49) by similar reduction of the aldehyde (48). Formation of aldehyde (48) and epimerization to give (47) is also possible, but the reverse process is not (results discussed later). Further elaboration of trachelanthamidine (31) affords retronecine (1), heliotridine (50), and otonecine (41); whereas rosmarinecine (51) can be formed from isoretronecanol (49) *via* platynecine (40).

3 Stereochemistry of Enzymic Processes Involved in Necine Biosynthesis

The initial step in the biosynthetic pathway (Scheme 15) received the first attention with regard to stereochemistry. Three independent groups of workers, employing different strategies, all showed that decarboxylation of L-ornithine (4) and L-arginine (6) takes place with retention of configuration.[39] This was an important finding, because it provided a method for the preparation of putrescines enantiomerically deuteriated at C-1 (*vide infra*).

In order to provide further information about the stereochemistry of the enzymic processes contained in Scheme 15, it was necessary to use precursors specifically labelled with deuterium, and then establish complete labelling patterns in isolated alkaloids by ^2H n.m.r. spectroscopy. Initial experiments were carried out on *Senecio isatideus*, and so a complete assignment of the ^1H n.m.r. spectrum of retrorsine (3) was made using proton decoupling and n.O.e. experiments.[40] The first precursor used was [2,3-$^2H_4$]putrescine dihydrochloride, made by exchanging the protons in succinonitrile with ^2H_2O, followed by catalytic hydrogenation, and acidification. In all experiments with ^2H-labelled precursors, [1,4-^{14}C]putrescine dihydrochloride was fed as well, in order to provide an indication of the ^{14}C specific incorporation. This was then compared with the ^2H specific incorporation estimated from the ^2H n.m.r. spectrum by reference to the concentration of the sample and the ^2H natural abundance signal(s) in the n.m.r. solvent used. From the start of this work, ^2H-labelled precursors gave highly satisfactory specific incorporations of 3—5% per C_4 unit. However, $^2H\{^1H\}$ n.m.r. spectra of retrorsine were disappointing because of the

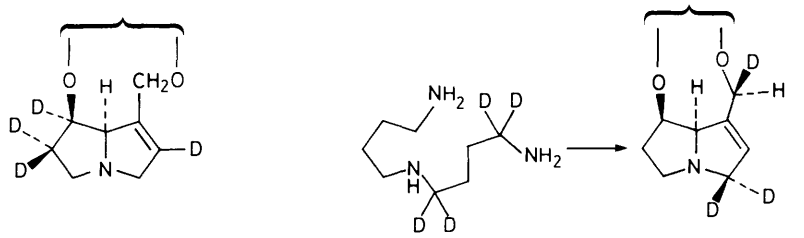
[39] G. R. Orr and S. J. Gould, *Tetrahedron Lett.*, 1982, **23**, 3139; I. D. Wigle, L. J. J. Mestichelli, and I. D. Spenser, *J. Chem. Soc., Chem. Commun.*, 1982, 662; D. J. Robins, *Phytochemistry*, 1983, **22**, 1133.
[40] J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1983, 1222.


NH2
NH2
H
CO2H
(4)
NH2
NH2
(5)
CHO
NH2
NH2
NH2
N
(45)
NH2
NH2
N
H
(21)
NH2
N
+
(29)
CHO
N
+
(46)
H
CHO
N
(48)
?
H
CHO
N
(47)
H
CH2OH
N
(31)
H
CH2OH
N
(49)
HO
H
CH2OH
N
(1)
HO
H
CH2OH
N
(50)
HO
H
CH2OH
N
(40)
HO
O
CH2OH
N
Me
(41)
HO
H
CH2OH
OH
N
(51)

Scheme 15

presence of very broad bands. Much narrower signals were obtained at higher temperatures, which were consequently employed throughout the rest of this work. Thus, the $^2H\{^1H\}$ n.m.r. spectrum of retrorsine (52) taken at 60 °C in chloroform after feeding [2,3-$^2H_4$]putrescine dihydrochloride to *S. isatideus* (Figure 8a) shows four sites of retrorsine about equally enriched with ^2H at the expected positions of H-2, H-6α, H-6β, and H-7α. The presence of ^2H at H-7α does show that introduction of the hydroxy group at this position does not involve keto or enol intermediates.[40]

The next obvious precursor to use was [1,4-$^2H_4$]putrescine dihydrochloride. This was made by catalytic hydrogenation of succinonitrile in ^2H_2O followed by acidification. When the $^2H\{^1H\}$ n.m.r. spectrum of retrorsine obtained after feeding this precursor to *S. isatideus* was run at 60 °C in chloroform (Figure 8b), signals were still quite broad, and narrower signals were obtained at 90 °C in pyridine (Figure 8c). The three major signals are due to retrorsine (54) labelled



(52) (53) (54)

with ^2H at H-3α, H-3β, and H-9 *pro-S*.[40] At first sight this is a surprising result with most of the ^2H located in one half of the base portion. This was explained by considering the most likely homospermidine intermediate (53) formed by reaction of labelled putrescine with endogenous unlabelled material. If this intermediate (53) is subject to a ^2H isotope effect during the oxidation to give the iminium ion, the unlabelled end of the homospermidine will be preferentially converted into an aldehyde, leading to the observed preponderance of the ^2H in one half of the base portion. The labelling of the H-9 *pro-S* with ^2H establishes that in the reduction of the aldehyde [(47)→(31)] a proton is added to the *re*-face of the carbonyl group. This is the usual stereochemistry associated with coupled dehydrogenase enzyme systems.[41]

More rigorous examination of this point became possible when (*R*)- and (*S*)-[1-^2H]putrescine were made enzymically using the known stereospecificity of L-ornithine decarboxylase. Richards and Spenser used the decarboxylation of L-ornithine in ^2H_2O with ornithine decarboxylase to generate (*R*)-[1-^2H]-putrescine, whereas similar decarboxylation of the L-component of [2-^2H]-DL-ornithine in water gave (*S*)-[1-^2H]putrescine.[42] The mode of incorporation of these precursors into a mixture of pyrrolizidine alkaloids in *Senecio vulgaris*

[41] R. Bentley, 'Molecular Asymmetry in Biology,' Academic Press, New York, 1970, Vol. 2.
[42] J. C. Richards and I. D. Spenser, *Can. J. Chem.*, 1982, **60**, 2810.

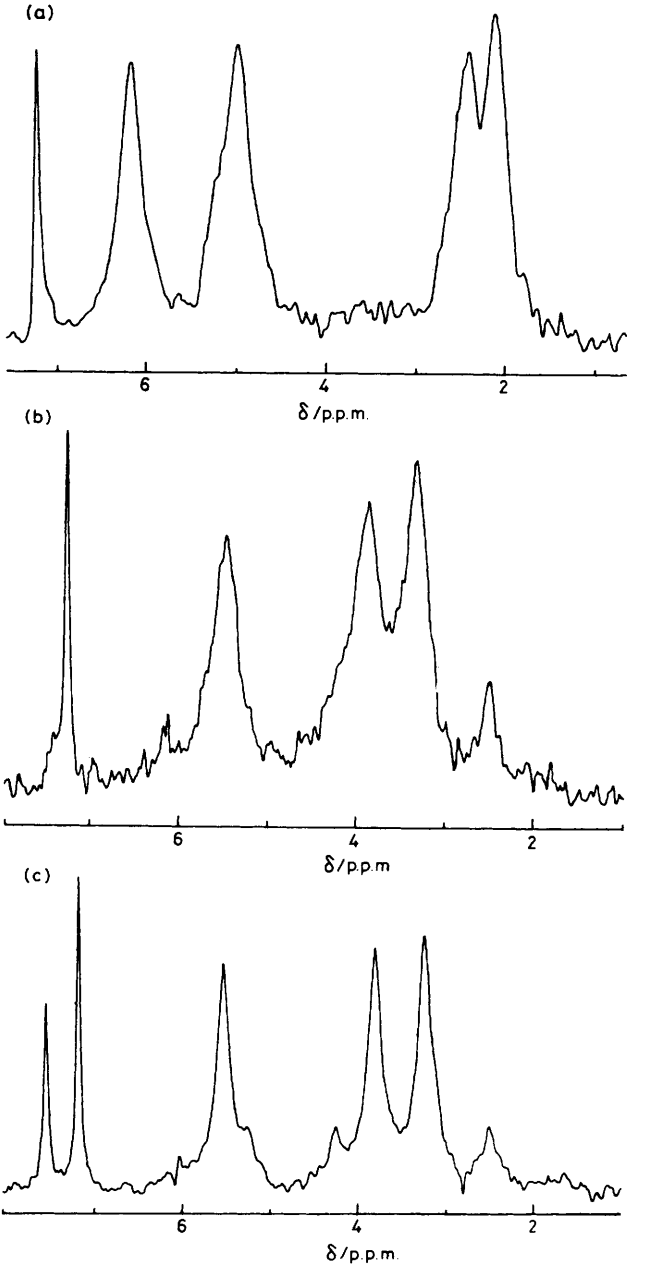


Figure 8 30.72 MHz $^2H\{^1H\}$ *N.m.r. spectrum of retrorsine* (3) (0.5 g): (*a*) *sample of* (52) *derived from* [2,3-$^2H_4$]*putrescine in* $CHCl_3$ *at* 60 °C; (*b*) *sample of* (54) *derived from* [1,4-$^2H_4$]*putrescine in* $CHCl_3$ *at* 60 °C; (*c*) *sample of* (54) *in pyridine at* 90 °C (*natural abundance signals for pyridine are at* δ 7.2 *and* 7.6 *p.p.m.*)

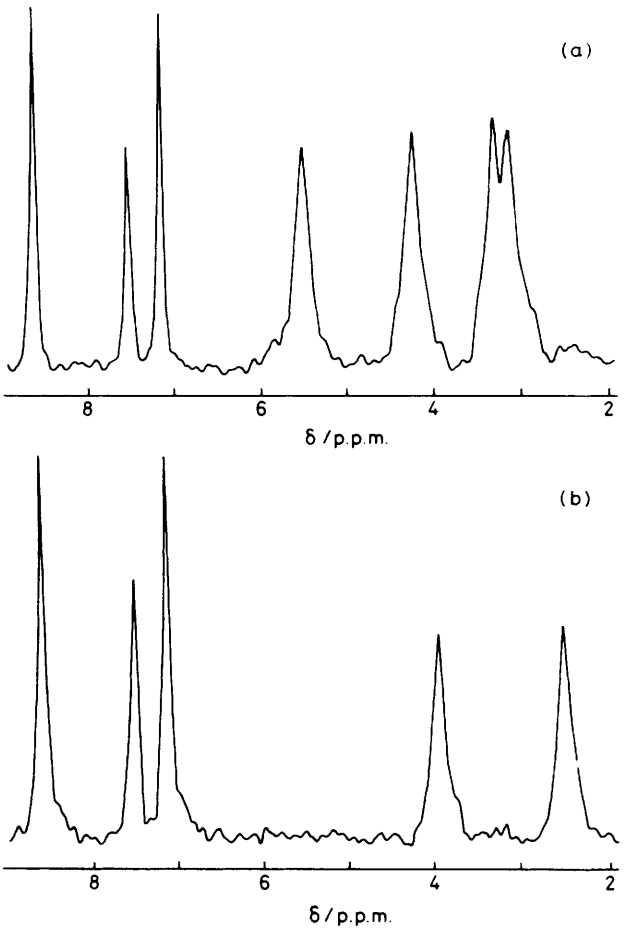
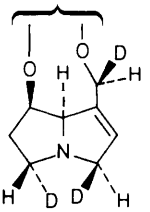


Figure 9 30.72 MHz $^2H\{^1H\}$ *N.m.r. spectra of retrorsine in pyridine at* 90 °C: (*a*) *sample of* (55) *derived from* (*R*)-[1-^{2}H]*putrescine;* (*b*) *sample of* (56) *derived from* (*S*)-[1-^{2}H]*putrescine. Natural abundance* ^2H *signals for pyridine are at* δ 7.2, 7.6, *and* 8.7 *p.p.m.*

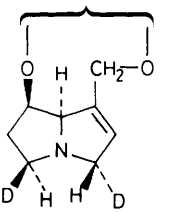
was determined.[43] For clarity, our results, obtained independently, are shown in Figure 9 for the $^2H\{^1H\}$ n.m.r. spectra of retrorsine obtained in pyridine at 90 °C.[44] After feeding (*R*)-[1-^{2}H]putrescine to *S. isatideus*, four equally labelled sites were observed in the $^2H\{^1H\}$ n.m.r. spectrum (Figure 9a) corresponding to retrorsine (55) labelled with ^2H at H-3β, H-5α, H-8α, and H-9 *pro-S*. This labelling pattern shows that no ^2H is lost from this precursor on conversion into

[43] G. Grue-Sorensen and I. D. Spenser, *J. Am. Chem. Soc.*, 1983, **105**, 7401.
[44] J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1984, 517; *J. Chem. Soc., Perkin Trans. 1*, 1986, 983.

retrorsine. This is consistent with the known stereospecificity of diamine oxidases in which the *pro-S* hydrogen is removed from the primary amino group.[45] This must apply to all three oxidations of the primary amino groups in the biosynthetic pathway to retronecine (1) (Scheme 15). Examination of the $^2H\{^1H\}$ n.m.r. spectrum obtained after feeding (*S*)-[1-^{2}H]putrescine to *S. isatideus* (Figure 9b) showed only two signals for H-3α and H-5β in retrorsine (56). This is



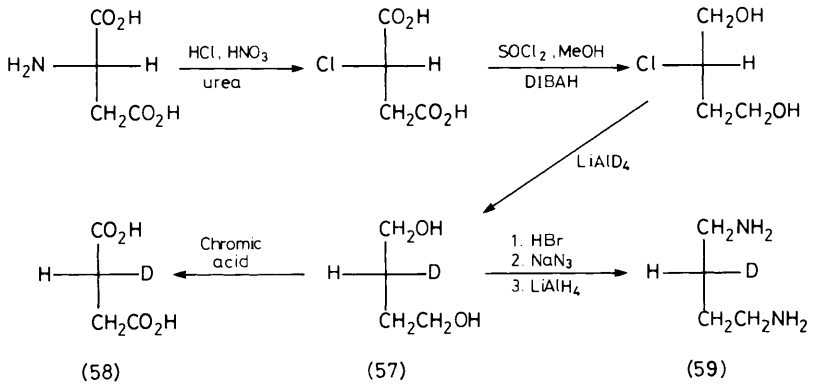
(55)



(56)

consistent with loss of ^2H on oxidation of each primary amino group. Moreover, reduction of the putative imine (45) must involve addition of a hydride donor to the *si*-face of the imine.

In order to complete the determination of stereochemical details possible in retronecine (1) biosynthesis, samples of putrescine enantiomerically deuteriated at C-2 were required. This was achieved by modifying an unpublished route to (*R*)-[2-^{2}H]succinic acid (58) by Arigoni and Eliel outlined in Scheme 16.[46] Some



(58) (57) (59)

Scheme 16

of the intermediate deuteriated diol (57) was oxidized to (*R*)-[2-^{2}H]succinic acid to provide comparison with literature data, and the remainder was converted into (*R*)-[2-^{2}H]putrescine (59) *via* the dibromide and diazide. The (*S*)-[2-

[45] A. R. Battersby, J. Staunton, and M. C. Summers, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1052.
[46] D. Arigoni and E. L. Eliel, *Top. Stereochem.*, 1969, **4**, 200.

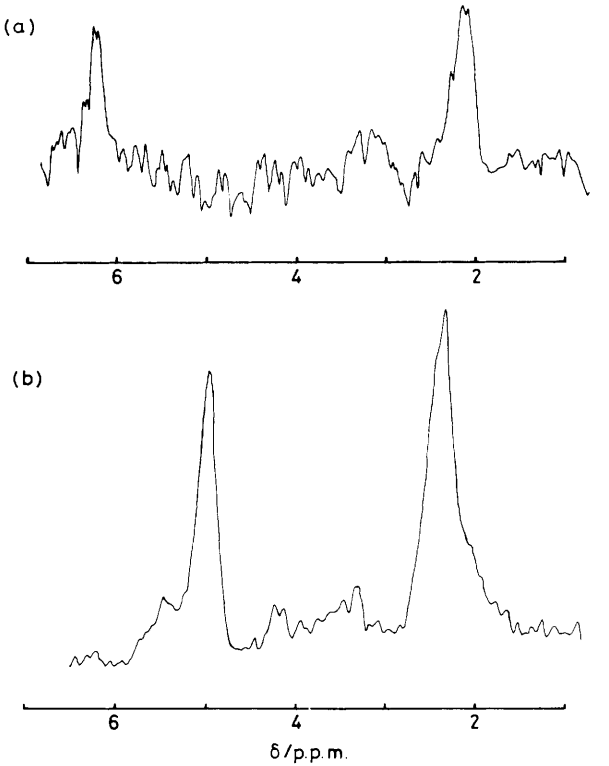
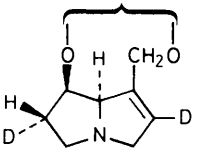


Figure 10 30.72 MHz $^2H\{^1H\}$ *N.m.r. spectra of retrorsine in chloroform at* 60 °C: (*a*) *sample of* (60) *derived from* (2*R*)-[2-^{2}H]*putrescine*; (*b*) *sample of* (61) *derived from* (2*S*)-[2-^{2}H]*putrescine. Natural abundance* ^{2}H *in* $CHCl_3$ *was the internal reference at* δ 7.15

^2H]putrescine was made in an analogous manner from (*R*)-aspartic acid.[47] Feeding experiments with *Senecio isatideus* were carried out as usual and the $^2H\{^1H\}$ n.m.r. spectra are shown in Figure 10. Two signals were visible in each spectrum. After feeding the (*R*)-isomer (59), ^{2}H was present at H-2 and H-6α, corresponding to the labelling pattern (60) in retrorsine. Incorporation of the (*S*)-isomer led to retrorsine (61) labelled with ^{2}H at H-6β and H-7α. These results show that formation of the double bond in retronecine (1) occurs with retention of the *pro-R* hydrogen and loss of the *pro-S* hydrogen at the carbon which becomes C-2 of retronecine. Furthermore, hydroxylation at C-7 of retronecine occurs with retention of configuration. This is the usual stereospecificity observed for direct hydroxylations at sp^3 carbon atoms.[41]

The availability of (*R*)- and (*S*)-forms of [1-^{2}H]- and [2-^{2}H]-putrescine enabled us to extend the studies to the stereochemistry of the enzymic processes

[47] E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1985, 1450; *J. Chem. Soc., Perkin Trans. 1*, 1987, 1089.



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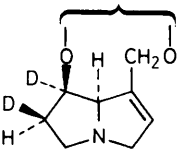
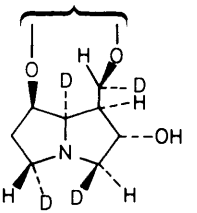
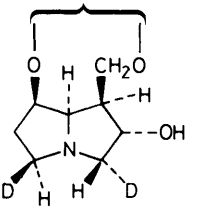


(61)

involved in the formation of rosmarinecine (51). Feeding experiments were carried out on *Senecio pleistocephalus* with these precursors and good ^{14}C specific incorporations of 3.7 to 9.2% per C_4 unit were observed in rosmarinine (28).[48] The ^1H n.m.r. spectrum of rosmarinine was fully assigned. The $^2H\{^1H\}$ n.m.r. spectra for rosmarinine obtained after feeding (*R*)- and (*S*)-[1-^{2}H]putrescine are shown in Figure 11 and correspond to labelling patterns (62) and (63),

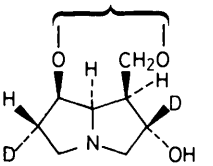


(62)

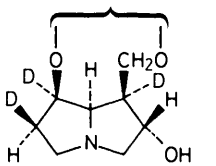


(63)

respectively in rosmarinine. These patterns are exactly analogous to those observed for retrorsine. Additional information was obtained from the $^2H\{^1H\}$ n.m.r. spectra of rosmarinine after feeding (*R*)- and (*S*)-[2-^{2}H]putrescine (Figure 12). These spectra give rise to the labelling patterns (64) and (65), respectively for



(64)



(65)

rosmarinine. The presence of ^2H at C-1 in rosmarinine (65) after feeding the (*S*)-isomer shows that the *pro-R* hydrogen is stereospecifically removed on formation of the pyrrolizidine ring. The presence of this ^2H is also consistent with the direct formation of the aldehyde (48) from iminium ion (46), rather than *via* the *exo*-aldehyde (47). [The alternative isomerization (48)→(47) is still a possibility in retronecine (1) biosynthesis (Scheme 15).] Both hydroxylations at C-2 and C-7 of

[48] H. A. Kelly and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1987, 2195.

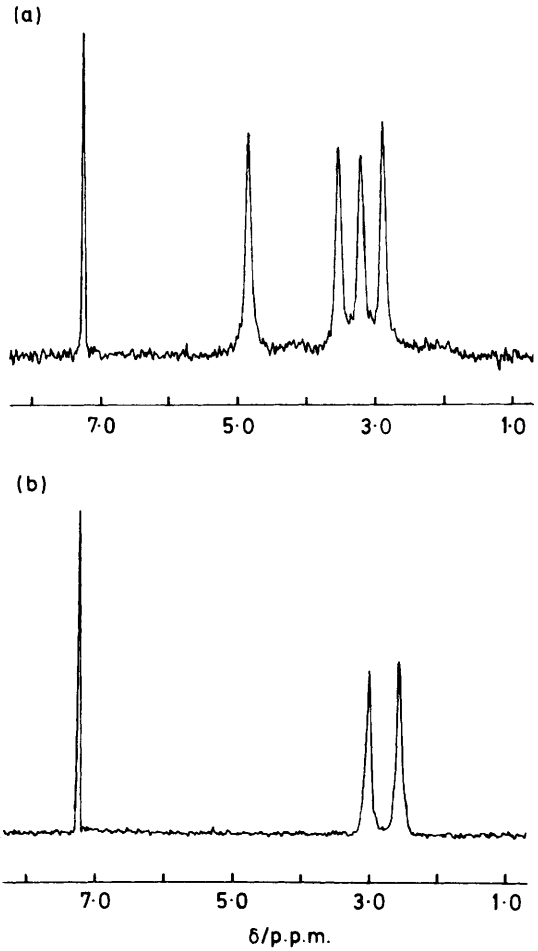


Figure 11 55.28 MHz $^2H\{^1H\}$ *N.m.r. spectra of rosmarinine in* $CHCl_3$ *at* 60 °C: (*a*) *sample of rosmarinine* (62) *derived from* (*R*)-[1-^{2}H]*putrescine;* (*b*) *sample of rosmarinine* (63) *derived from* (*S*)-[1-^{2}H]*putrescine. The signal at* δ 7.25 *is natural abundance* ^2H *in* $CHCl_3$

isoretronecanol (49) must proceed with retention of configuration to give rosmarinecine (51).[48]

The presence of the 1,2-double bond in pyrrolizidine alkaloids such as retrorsine (3) is necessary for observation of the hepatotoxic action. The introduction of the double bond in retronecine (1) may plausibly occur by hydroxylation at C-2α and C-7 of trachelanthamidine (31) with normal retention of configuration, followed by *trans*-elimination of the elements of water from the 1,2-position (Scheme 15). The alternative hydroxylation at C-1 is less likely for

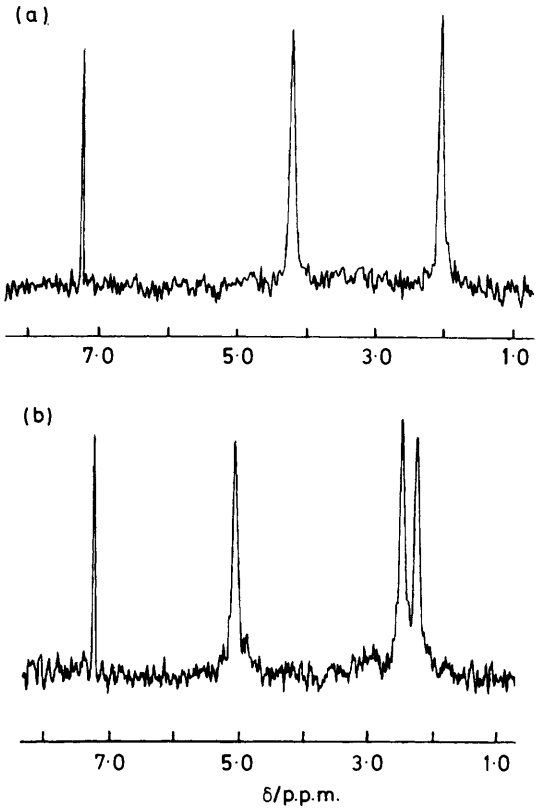


Figure 12 55.28 MHz $^2H\{^1H\}$ *N.m.r. spectra of rosmarinine in* $CHCl_3$ *at* 60 °C: (*a*) *sample of rosmarinine* (64) *derived from* (*R*)-[2-^{2}H]*putrescine*; (*b*) *sample of rosmarinine* (65) *derived from* (*S*)-[2-^{2}H]*putrescine. The signal at* δ 7.25 *is natural abundance* ^2H *in* $CHCl_3$

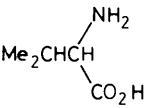
circumstantial reasons—a number of necines occur with hydroxyl groups at C-2, whereas only one is claimed to possess a hydroxyl group at C-1 of the necine.[1] It is interesting to note that formation of a double bond from rosmarinecine (51) would require *cis*-elimination of the elements of water.

The enantiomerically deuteriated putrescines will be used to determine the stereochemistry of the enzymic processes involved in the formation of other necines such as otonecine (41), heliotridine (50), and bases with 8β stereochemistry.

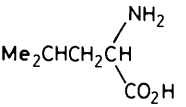
4 Necic Acids

The necic acids exhibit a great range of structural types.[1] Most are C_{10} diacids [as in retrorsine (3)] which at first sight appeared to be terpenoid in origin. However, the extent of oxygenation and mode of coupling of the two C_5 units

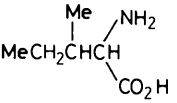
were not normal for monoterpenoids. Indeed, feeding of [2-^{14}C]mevalonolactone to *Senecio isatideus* produced inactive retrorsine (3).[49] Partial labelling patterns were determined by degradation of alkaloids after feeding ^{14}C-labelled acetates to *Senecio* species, but these were difficult to interpret.[49–51] Many experiments with ^{3}H- and ^{14}C-labelled precursors have shown that all the necic acids so far studied are derived from common α-amino acids, namely valine (66), leucine (67), isoleucine (68), and threonine (69).



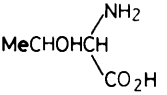
(66)



(67)



(68)

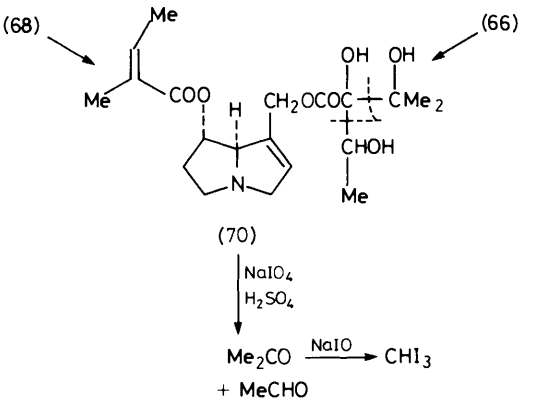


(69)

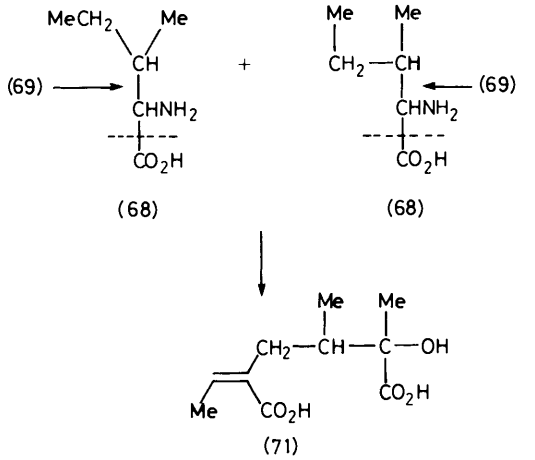
Crout studied the biosynthesis of the two necic acids of heliosupine (70) in *Cynoglossum officinale*. He showed that DL-[4-^{14}C]valine was incorporated efficiently into echimidinic acid, and most of the radioactivity was present in the acetone and iodoform produced by degradation (Scheme 17).[52] Valine presumably provides five of the carbon atoms of echimidinic acid with two additional carbons being added during the biosynthesis. On the other hand, L-[U-^{14}C]isoleucine was specifically incorporated into the angelic acid portion of heliosupine (70).[53] Isoleucine is also known to label the tigloyl ester portion of tropane alkaloids in *Datura meteloides*.[54] The incorporation of [1-^{14}C]tiglic acid into the angelic acid portion of heliosupine (70) suggests that isomerization can occur during the formation of the alkaloid.[55]

Most studies in this area have been carried out on senecic acid (71) by Crout and his co-workers with *Senecio magnificus* which produces senecionine (7). It has emerged that senecic acid is formed from two molecules of isoleucine (68) [or its biosynthetic precursor threonine (69)] with loss of both carboxyl carbons of isoleucine (Scheme 18).[50,56] It was further shown that L-isoleucine is the only one

[49] C. Hughes and F. L. Warren, *J. Chem. Soc.*, 1962, 34.
[50] D. H. G. Crout, M. H. Benn, H. Imaseki, and T. A. Geismann, *Phytochemistry*, 1966, **5**, 1.
[51] C. G. Gordon-Gray and F. D. Schlosser, *J. S. Afr. Chem. Inst.*, 1970, **23**, 13.
[52] D. H. G. Crout, *J. Chem. Soc.*, (*C*), 1966, 1968.
[53] D. H. G. Crout, *J. Chem. Soc.*, (*C*), 1967, 1233.
[54] W. C. Evans and J. G. Woolley, *J. Pharm. Pharmacol.*, 1965, **17**, suppl., 37S.
[55] B. A. McGaw and J. G. Woolley, *Phytochemistry*, 1979, **18**, 1647.
[56] D. H. G. Crout, N. M. Davies, E. H. Smith, and D. Whitehouse, *J. Chem. Soc., Chem. Commun.*, 1970, 635; *J. Chem. Soc., Perkin Trans. 1*, 1972, 671.



Scheme 17



Scheme 18

of the four possible stereoisomers of isoleucine that is incorporated well into senecic acid.[57]

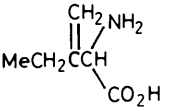
Attempts to identify the five-carbon precursor of senecic acid (71) were carried out by Crout and co-workers. Negative results were obtained with angelate, 2-methylbutanoate, and 2-methyl-3-oxobutanoate.[58]

Experiments with isoleucine (68) stereospecifically labelled with ^{3}H at C-4

[57] N. M. Davies and D. H. G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2079.
[58] N. M. Bale, R. Cahill, N. M. Davies, M. B. Mitchell, E. H. Smith, and D. H. G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1978, 101.

have shown that H-4 *pro-S* is lost and H-4 *pro-R* is retained from both molecules required to form senecic acid (71).[59]

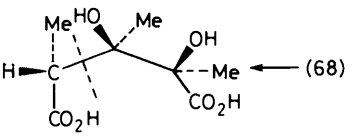
There still remains the intriguing question as to how the two units derived from isoleucine (68) are joined from C-4 of one isoleucine to C-6 of the other to form senecic acid (71) (Scheme 18). Crout and co-workers suggested that one activated intermediate might be β-methylenenorvaline (72), which could be



(72)

formed from isoleucine by dehydrogenation. Experiments with β-[$^3H_2$]methylenenorvaline on *Senecio magnificus* showed that it is incorporated efficiently into senecic acid (71). Unfortunately, degradations could not be carried out to see if one or both halves of the senecic acid were labelled.[58] These results, taken together with the negative ones obtained with potential C_5 precursors, suggest that the activation steps (and perhaps the coupling step) for the two portions may occur at the C_6 level.

Other work on the biosynthesis of necic acids has been carried out on *Crotalaria* species. Crout and co-workers showed that L-[U-^{14}C]isoleucine and L-[U-^{14}C]threonine were both specifically incorporated into the monocrotalic acid (73) portion of monocrotaline (2) in *C. retusa*.[60] The results from partial



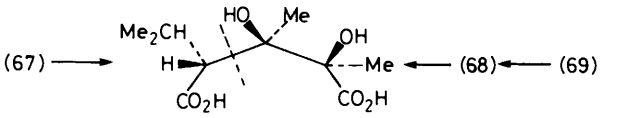
(73)

degradations of labelled monocrotalic acid were consistent with the derivation of a C_5 unit from isoleucine (similar to that used in senecic acid biosynthesis), while the origin of the remaining three carbon atoms of monocrotalic acid remains uncertain. Devlin and Robins studied the biosynthesis of trichodesmic acid (74), the acid portion of trichodesmine, present in *C. globifera*.[61] The ^{14}C-labelled amino acids threonine (69), isoleucine (68), valine (66), and leucine (67) were all

[59] R. H. Cahill, D. H. G. Crout, M. B. Mitchell, and U. S. Muller, *J. Chem. Soc., Chem. Commun.*, 1980, 419; R. Cahill, D. H. G. Crout, M. V. M. Gregorio, M. B. Mitchell, and U. S. Muller, *J. Chem. Soc., Perkin Trans. 1*, 1983, 173.

[60] D. J. Robins, N. M. Bale, and D. H. G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2082.

[61] J. A. Devlin and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1329.



(74)

incorporated specifically into trichodesmic acid (74). Partial degradations of the labelled trichodesmic acid samples showed that threonine and isoleucine label the right-hand half of trichodesmic acid (identical to that in monocrotalic acid), whereas valine and leucine are mainly incorporated into the left-hand portion of trichodesmic acid (74).

Further information about the formation of the necic acids and how they join with necines to be converted into macrocyclic alkaloids is likely from the application of precursors labelled with stable isotopes. It is known that the alkaloids are often stored as *N*-oxides.[62]

Progress in determining details of the biosynthetic pathways to a range of pyrrolizidine alkaloids has been rapid over the past few years, particularly because of the availability of precursors labelled with stable isotopes, used in conjunction with the powerful technique of n.m.r. spectroscopy to establish complete labelling patterns. Some information *e.g.* from use of ^{13}C–^{15}N doubly labelled precursors, has been obtained which would be impossible to produce from use of radiotracers. Careful synthesis of enantiomerically deuteriated precursors has been used to probe the stereochemical details of enzymic processes involved in the biosynthetic pathways. The use of isolated enzymes to carry out steps in the biosynthetic sequence is an area worthy of further attention.

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[62] T. Hartmann and G. Toppel, *Phytochemistry*, 1987, **26**, 1639.