Pyrrolizidine Alkaloid Biosynthesis; Incorporation of ^{2}H-Labelled Putrescines into Retrorsine

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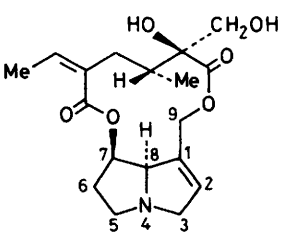
The labelling patterns in retrorsine (**1**) derived biosynthetically from [1,4-$^2H_4$]- and [2,3-$^2H_4$]-putrescine have been established by ^{2}H n.m.r. spectroscopy; in the former case the formation of (9*S*)-[9-^{2}H]retrorsine (**12**) is consistent with stereospecific addition of hydrogen to the *re*-face of an aldehyde precursor (RC^{2}HO).

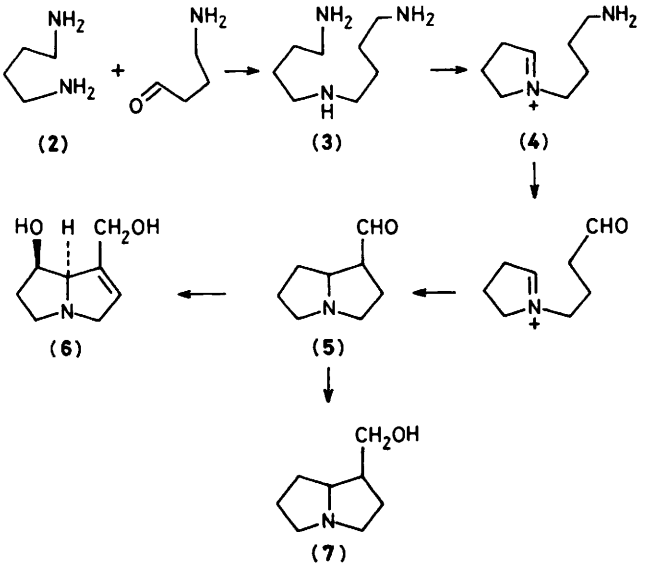
Retronecine (**6**) is the base portion of many pyrrolizidine alkaloids[1,2] including retrorsine (**1**), which is the major alkaloid present in *Senecio isatideus* plants. Retronecine is derived biosynthetically from L-ornithine or L-arginine[3] *via* putrescine (**2**).[4–7] It has been shown that homospermidine (**3**), formed from two molecules of putrescine, is a later intermediate in the biosynthetic pathway to retronecine.[6,8] An attempt was recently made to monitor the fate of hydrogen atoms in retronecine biosynthesis by using ornithines specifically labelled with ^{3}H.[9] Samples of retronecine labelled from [4-^{3}H]- and [5-^{3}H]-ornithine were degraded to obtain partial labelling patterns. The uncertainty in the distribution of ^{3}H due to the incompleteness of the degradation pathways and the lack of purity of one of the precursors limit the usefulness of these experiments. The high total incorporations obtained by us for ^{14}C-labelled precursors into retronecine[4] suggested that more convincing evidence for the fate of hydrogen atoms in the biosynthetic pathway could be obtained by the use of ^{2}H-labelled precursors in conjunction with ^{2}H n.m.r. spectroscopy on the retrorsine produced. We report the use of ^{2}H n.m.r. spectroscopy to establish complete labelling patterns in retrorsine (**1**) after feeding specifically ^{2}H-labelled putrescines to *S. isatideus* plants.



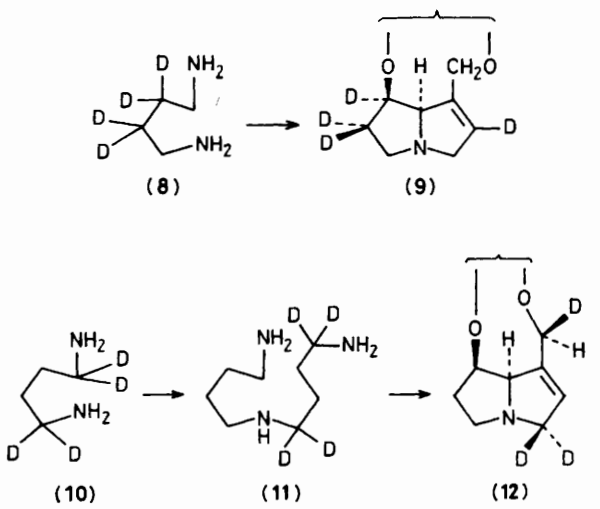
(**1**)

Catalytic hydrogenation of [2,3-$^2H_4$]succinonitrile (containing >99% $^2H_4$ species) gave [2,3-$^2H_4$]putrescine (**8**), which was isolated and recrystallised as the dihydrochloride (60% yield) [$^2H\{^1H\}$ n.m.r. (H_2O) δ 1.76 p.p.m. (s)]. Similar catalytic reduction of succinonitrile under a deuterium atmosphere yielded [1,4-$^2H_4$]putrescine (**10**) (containing >95% $^2H_4$ species), which was converted into the dihydrochloride [$^2H\{^1H\}$ n.m.r. (H_2O) δ 3.07 p.p.m. (s)].

Pulsed feeding of each precursor (0.25—0.5 g) was carried out by direct absorption of sterile aqueous solutions into the xylems of mature *Senecio isatideus* plants through stem



Scheme 1



punctures on alternate days during a three week period.[4] [1,4-$^{14}C_2$]Putrescine dihydrochloride (10 μCi) was added to each ^{2}H-labelled precursor. After a further two weeks the plants were harvested and retrorsine was isolated and recrystallised to constant specific radioactivity. Specific incorporations of 3—5% ^{14}C were obtained for retrorsine. The ^{2}H{^{1}H} n.m.r. spectra of these samples in chloroform displayed very broad bands. It was found that increased temperatures produced much narrower signals.[10] Thus, with [2,3-$^2H_4$]putrescine as precursor, the ^{2}H{^{1}H} n.m.r. spectrum of retrorsine in chloroform at 60 °C [Figure 1(a)] showed four signals at δ 2.15, 2.40, 5.0, and 6.20 p.p.m., corresponding to retrorsine (**9**) labelled with ^{2}H at C-6α, C-6β, C-7α, and C-2.† The enrichment factors‡ for each labelled site in retrorsine are approximately equal at *ca.* 1.9%. Thus the specific incorporation of ^{2}H for two units of putrescine into retrorsine is *ca.* 3.8%. The labelling pattern observed is that expected for incorporation of two putrescine molecules into retronecine *via* the symmetrical intermediate homospermidine (**3**). The presence of ^{2}H at C-7α confirms that the introduction of the hydroxy group at this position does not involve a keto or enol intermediate.[9]

The ^{1}H and ^{2}H{^{1}H} n.m.r. spectra of retrorsine derived from [1,4-$^2H_4$]putrescine (**10**) were both obtained in chloroform at 60 °C and compared. It was clear that three main signals were present at δ 3.35, 3.90, and 5.45 p.p.m. [Figure 1(b)], corresponding to 3β-H, 3α-H, and 9-H (*pro-S*) in retrorsine (**12**).† The enrichment factors for these three sites are approximately equal at *ca.* 1.5% ^{2}H. Observation of the ^{2}H{^{1}H} n.m.r. spectrum of this sample at 90 °C in pyridine [Figure 1(c)] gave sharper signals and allowed smaller signals at δ 2.50 and 4.26 p.p.m. due to 5β-H and 8-H to be distinguished, with enrichment factors of *ca.* 0.3% ^{2}H. (The possible presence of a signal for 5α-H at δ 3.25 p.p.m. was obscured by the large resonance due to 3β-H.) The specific incorporation for two putrescine units is thus *ca.* 1.8% ^{2}H, but somewhat surprisingly, most of the ^{2}H is located in the right-hand half of the base portion [as in (**12**)].

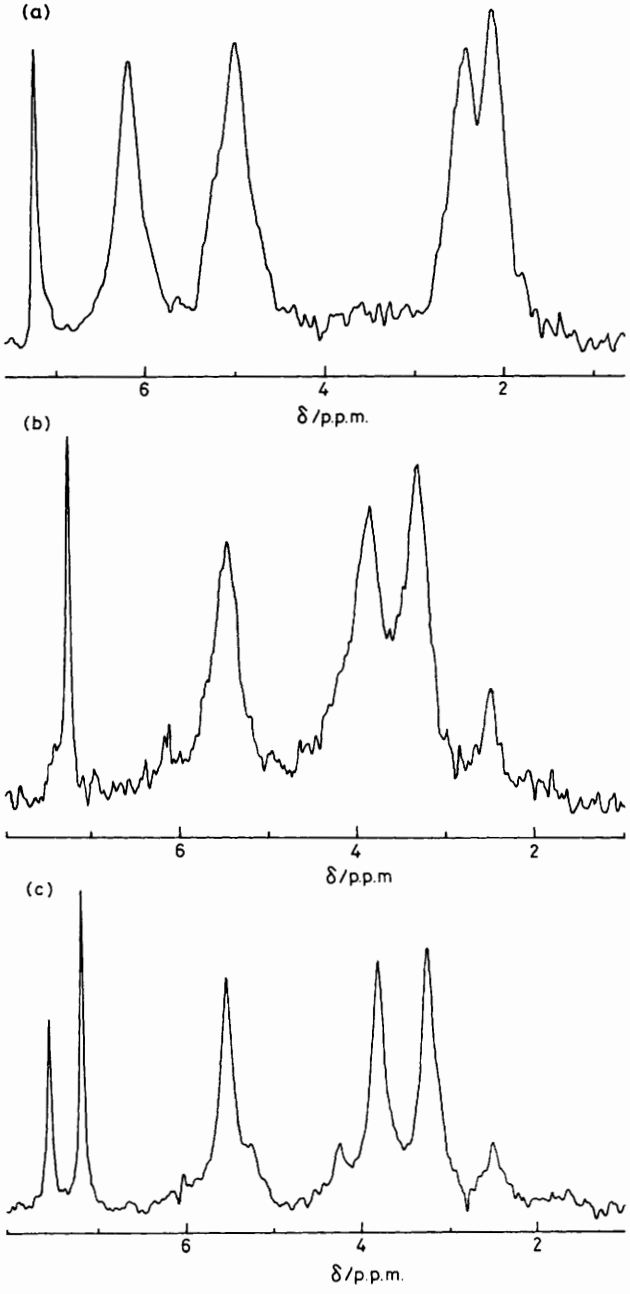


Figure 1. 30.72 MHz ^{2}H{^{1}H} N.m.r. spectrum of retrorsine (**1**) (0.5 g): (a) sample of (**9**) derived from [2,3-$^2H_4$]putrescine (**8**) in $CHCl_3$ at 60 °C; (b) sample of (**12**) derived from [1,4-$^2H_4$]putrescine (**10**) in $CHCl_3$ at 60 °C; (c) sample of (**12**) in pyridine at 90 °C (natural abundance signals for pyridine are at δ 7.2 and 7.6 p.p.m.).

A possible explanation for this result follows from consideration of the proposed biosynthetic pathway to retronecine (**6**) (Scheme 1). Initial oxidation of putrescine (**2**) by diamine oxidase or transaminase enzymes yields 4-aminobutanal which can couple with another molecule of putrescine to give homospermidine (**3**) after reduction of the imine. Further sequential oxidation of the primary amino groups in

† The 360 MHz ^{1}H n.m.r. spectral data for retrorsine (**1**) in $CDCl_3$ were assigned using extensive ^{1}H decoupling and nuclear Overhauser enhancement experiments: δ (retronecine portion only): 2.15 (1H, m, 6α-H), 2.38 (1H, dd, 6β-H), 2.53 (1H, m, 5β-H), 3.25 (1H, t, 5α-H), 3.38 (1H, m, 3β-H), 3.93 (1H, dd, 3α-H), 4.09 (1H, d, 9-H *pro-R*), 4.26 (1H, m, 8α-H), 5.00 (1H, t, 7α-H), 5.50 (1H, d, 9-H *pro-S*), 6.20 (1H, d, 2-H). The coupling constants (Hz) are: $J_{2,3\alpha}$ 1.8, $J_{2,3\beta}$ 1.7, $J_{2,8\alpha}$ 1, $J_{3\alpha,3\beta}$ 15.8, $J_{3\alpha,8\alpha}$ 1.8, $J_{3\beta,8\alpha}$ 6.2, $J_{5\alpha,5\beta}$ 9, $J_{5\alpha,6\alpha}$ 8.3, $J_{5\beta,6\alpha}$ 12, $J_{5\beta,6\beta}$ 5.8, $J_{6\alpha,6\beta}$ 13.8, $J_{6\alpha,7\alpha}$ 4, $J_{7\alpha,8\alpha}$ 4, $J_{9pro\text{-}R,9pro\text{-}S}$ 11.8.

‡ The enrichment factor for a labelled site in retrorsine is calculated from (integral of labelled site in retrorsine/concentration of retrorsine)/(natural abundance integral of ^{2}H in $CHCl_3$ at δ 7.25 p.p.m./concentration of $CHCl_3$) × 0.0156%.

homospermidine will lead to the aldehyde (**5**) by Mannich reaction. The recent synthesis[11] of the pyrrolizidine alkaloid trachelanthamidine (**7**) from homospermidine (**3**) using a diamine oxidase and physiological conditions to produce (**5**), followed by reduction with a coupled dehydrogenase system is support for this pathway. If the initial oxidation of putrescine (**2**) to 4-aminobutanal is subject to a substantial ^2H isotope effect, then formation of [$^2H_3$]-4-aminobutanal will be slow, and the most likely homospermidine species to be produced will be (**11**), formed by reaction of [1,4-$^2H_4$]-putrescine (**10**) with endogenous unlabelled 4-aminobutanal. If this intermediate (**11**) is also subject to a ^2H isotope effect in the next oxidation step, the unlabelled end of the homospermidine will be preferentially converted into an aldehyde leading to the unsymmetrical intermediate (**4**) preferentially deuteriated in the side chain. Thereafter retronecine will be formed with most of the ^2H in the right hand portion [as in (**12**)]. Thus, the labelling pattern observed is consistent with the proposed biosynthetic pathway.

Furthermore, the formation of (9*S*)-[9-^2H]retrorsine (**12**)† suggests that in the reduction of the aldehyde precursor RC^{2}HO, the ^1H is added to the *re*-face of the carbonyl group (this assumes that subsequent ester formation occurs with retention of configuration at C-9 of retronecine). The stereospecificity observed is that expected from a normal coupled dehydrogenase enzyme system.[12]

More insight into the stereochemistry of the enzyme processes involved in retronecine biosynthesis is likely from the use of other ^2H-labelled precursors.

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