^{2}H N.M.R. Spectroscopy as a Probe of the Stereochemistry of Biosynthetic Reactions. The Biosynthesis of Nicotine

Ian D. Wigle, Louis J. J. Mestichelli, and Ian D. Spenser

Department of Chemistry, McMaster University, Hamilton, Ontario, Canada L8S 4M1

The pyrrolidine ring of nicotine is derived from L-ornithine which, in intact *Nicotiana tabacum*, is decarboxylated with retention of configuration; the resulting putrescine is *N*-methylated and then oxidised, with loss of the *pro-S* proton on C-4, to yield *N*-methyl-1-pyrrolinium ion, which is attacked by the pyridine ring precursor at the 1-*si*,2-*re* face to yield (*S*)-nicotine.

The accepted route from ornithine[1,2] (**1**) into the pyrrolidine ring of nicotine (**7**)[3,4] *via* putrescine (**2**),[5] *N*-methylputrescine (**3**),[6] *N*-methyl-4-aminobutanal (**4**)[7] and *N*-methyl-l-pyrrolinium ion (**5**),[3] which is based on the results of tracer experiments

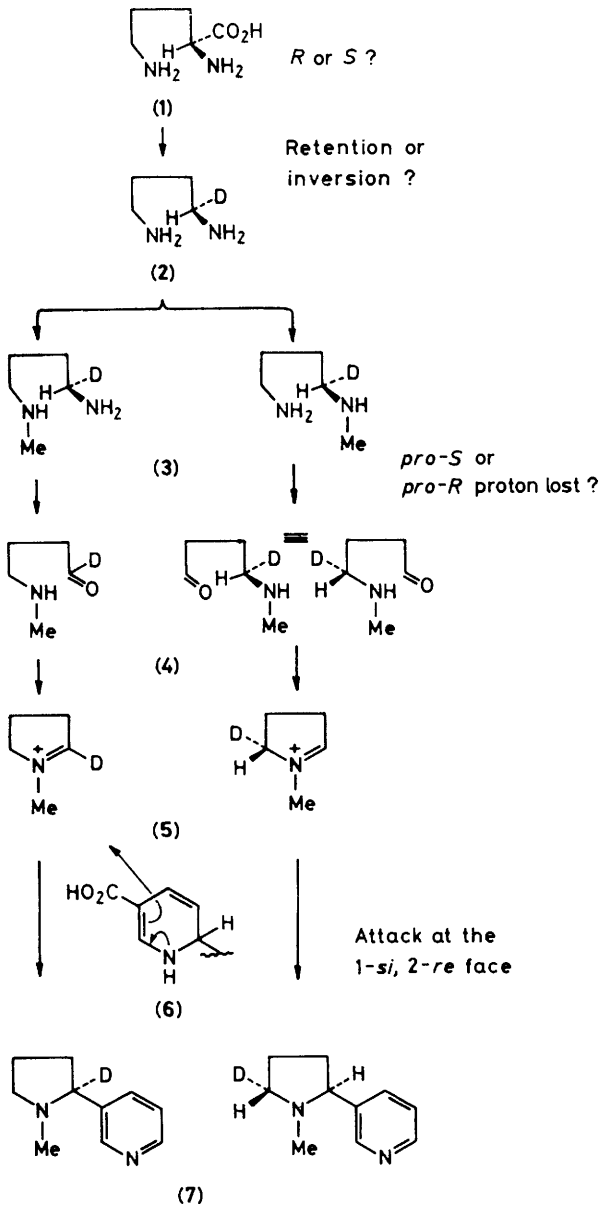
with intact plants[1–3,5,6] and tissue cultures[7] of *Nicotiana tabacum*, and on enzyme studies,[8,9] fully accounts for the origin of the pyrrolidine nucleus, in chemically rational terms. Stereochemical aspects of the biosynthetic sequence have so far not received attention.

We have employed ^{2}H n.m.r. spectroscopy to resolve one of the stereochemical ambiguities of the pathway, the prochirality of the conversion of *N*-methylputrescine (**3**) into *N*-methyl-4-aminobutanal (**4**).

(*R*)-[1-^{2}H]Putrescine dihydrochloride (**2**) (125 mg) was obtained by hydrolysis of (*R*)-[1-^{2}H]agmatine which had been prepared[10] by enzymic decarboxylation of L-arginine in ^2H_2O, a reaction which proceeds with retention of configuration.[10] A solution containing the (*R*)-[1-^{2}H]putrescine, together with [1,4-^{14}C]putrescine (68 μCi) (New England Nuclear), was administered to 12 3-month-old tobacco plants over a period of 15 days, by the wick method. Nicotine was isolated and purified and a small sample was converted into the diperchlorate for determination of radioactivity.† From the observed specific incorporation of ^{14}C (1.3%)‡ a deuterium enrichment corresponding to *ca.* 40 times natural abundance at deuteriated position(s) was inferred.‡

The ^{2}H n.m.r. spectrum§ (Figure 1) showed that deuterium was present at two positions of the pyrrolidine ring of nicotine, to an approximately equal extent. The chemical shifts¶ of the two ^{2}H n.m.r. signals corresponded to those of the 5′-*pro*-*R*-proton (δ 2.33 ± 0.02 p.p.m.)** and of the 2′-proton (δ 3.10 ± 0.02 p.p.m.) of nicotine, respectively. (Literature[11] values: 5′-*pro*-*R*, δ 2.31 ± 0.02; 2′-, δ 3.07 ± 0.02; 5′-*pro*-*S*, δ 3.25 ± 0.02 p.p.m.)

From this result the stereochemical course of the incorpora-



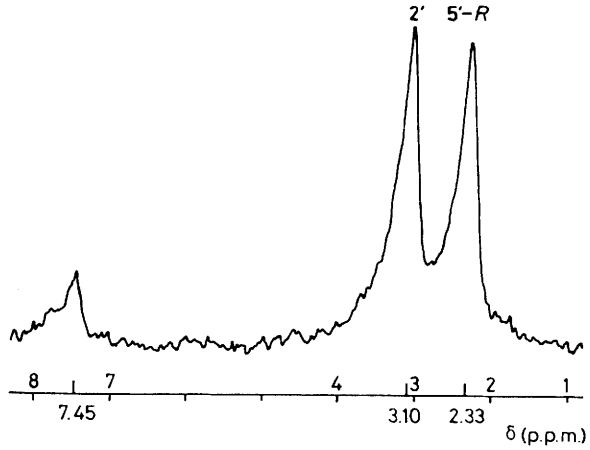


Figure 1. 61.42-MHz ^{2}H N.m.r. spectrum of nicotine (273 mg, in chloroform, 1.1 ml) isolated from tobacco plants to which *R*-[1-^{2}H]putrescine had been administered. The signal at δ 7.45 p.p.m. is due to natural abundance C[^{2}H]Cl$_3$ in the solvent.

† Radioactivity was assayed by liquid scintillation counting (Beckman LS 9000 Liquid Scintillation System). All samples were recrystallized to constant specific radioactivity, dissolved in Aquasol (New England Nuclear), and counted in triplicate, under comparable conditions of quenching.

‡ $\frac{\text{Molar specific activity of nicotine (dpm/mmol)}}{\text{Molar specific activity of putrescine (dpm/mmol)}} \times 100 = \frac{2.4 \times 10^6}{1.9 \times 10^8} \times 10^2 = 1.3$

$\frac{\%\text{ Specific incorporation} \times \frac{1}{2}}{\%\text{ Natural abundance of deuterium}} = \frac{1.3 \times \frac{1}{2}}{0.0156} = 42$ times natural abundance ^{2}H per enriched position.

§ The ^{2}H n.m.r. spectrum was recorded at 61.42 MHz in the Fourier mode on a Bruker-WM400 spectrometer (Southwestern Ontario NMR Centre, funded by a Major Installation grant from the Natural Sciences and Engineering Research Council of Canada, and located at the University of Guelph, Guelph, Ontario). The spectrum was determined in $CHCl_3$ with external ^2H_2O serving as frequency lock: acquisition time, 1.638 s; pulse width 30 μs. Natural abundance C[^{2}H]Cl$_3$ served as an internal standard.

¶ The chemical shift of $CHCl_3$ was found to change by as much as 0.5 p.p.m. as a function of nicotine concentration. Consequently the chemical shift of $CHCl_3$ relative to Me_4Si in the sample was determined in a separate experiment on a Varian EM 390 spectrometer.

** The observation that the 5′-*pro*-*R*-proton but not the 5′-*pro*-*S*-proton of nicotine was replaced by deuterium serves as independent evidence that the starting [1-^{2}H]putrescine was (*R*)-[1-^{2}H]-putrescine, that the [1-^{2}H]agmatine from which it was obtained was (*R*)-[1-^{2}H]agmatine, and that the reaction, catalysed by bacterial L-arginine decarboxylase, by which the agmatine was obtained, proceeds with retention of configuration.

tion of putrescine into nicotine may be inferred. Putrescine, a non-dissymmetric (C_{2v}) molecule, will be methylated at either nitrogen atom to give *N*-methylputrescine. Methylation of (*R*)-[1-^{2}H]putrescine will then give rise to an equimolar mixture of *N*-methyl-(*R*)-[1-^{2}H]putrescine and *N*-methyl-(*R*)-[4-^{2}H]putrescine.

Oxidative deamination of *N*-methyl-(*R*)-[1-^{2}H]putrescine, followed by cyclization, generates *N*-methyl-(*R*)-[5-^{2}H]-1-pyrrolinium ion as the sole product. This structure is known not to tautomerize under biosynthetic conditions.[3] *N*-Methyl-(*R*)-[5-^{2}H]-1-pyrrolinium ion in turn leads to nicotine labelled with deuterium in the 5′-*pro*-*R*-position.

Oxidative deamination of *N*-methyl-(*R*)-[4-^{2}H]putrescine, followed by cyclization, can lead either to *N*-methyl-1-pyrrolinium ion or to *N*-methyl-[2-^{2}H]-1-pyrrolinium ion, depending upon whether the *pro*-*R* or *pro*-*S* hydrogen, respectively, is stereospecifically lost during the oxidation. These two species would, in turn, give rise to unlabelled nicotine or to [2′-^{2}H]nicotine, respectively.

The nicotine generated in the course of the feeding experiment with (*R*)-[1-^{2}H]putrescine will be a mixture of several molecular species. The observed deuterium distribution of the isolated sample of nicotine permits inferences to be drawn about the mode of derivation of these species. Thus, if oxidation of *N*-methylputrescine proceeds with stereospecific loss of the *pro*-*R* hydrogen atom, deuterium will be incorporated into only one position in nicotine (5′-*pro*-*R*). Stereospecific loss of the *pro*-*S* hydrogen, on the other hand, will result in nicotine labelled with deuterium in two positions (5′-*pro*-*R* and 2′).

The results (Figure 1) show that two positions in nicotine, 5′-*pro*-*R* and 2′, are enriched in deuterium. It follows that it is the *pro*-*S* proton which is lost from the -CH_2NH_2 group of putrescine in the course of its conversion into nicotine. This is consistent with the known stereospecificity of reactions catalysed by diamine oxidase.[12–14]

Further experiments, with radioactive tracers, resolved the remaining stereochemical ambiguities of the route from ornithine into nicotine. Whereas the nicotine, isolated from a feeding experiment with a mixture of DL-[(*RS*)-5-^{3}H]ornithine and DL-[5-^{14}C]ornithine, showed a ^{3}H/^{14}C ratio which was approximately 0.7 times that of the doubly labelled ornithine that served as the substrate (calculated value, 0.75 times), the nicotine, isolated from an experiment with a mixture of L-[(*RS*)-5-^{3}H]ornithine and DL-[5-^{14}C]ornithine, showed a ^{3}H/^{14}C ratio which was 1.4 times that of the administered tracer. This demonstrates[15] that L-ornithine (predicted ^{3}H/^{14}C ratio of nicotine, 1.5 times that of substrate) rather than D-ornithine (predicted ^{3}H/^{14}C ratio of nicotine, zero) is the precursor of the pyrrolidine ring of nicotine.

Furthermore, a feeding experiment with intermolecularly doubly labelled DL-[2-^{3}H,5-^{14}C]ornithine[16] yielded a sample of nicotine whose ^{3}H/^{14}C ratio was approximately half that of the labelled substrate. Thus, only one tritium per two ^{14}C atoms entered the product. The ^{2}H-n.m.r. experiment shows that the *pro*-R proton at C-1 of putrescine is retained and the *pro*-*S* proton is lost in the course of nicotine biosynthesis. Thus, when L-ornithine (**1**) is converted into putrescine (**2**) *en route* into the pyrrolidine ring of nicotine (**7**), the α-proton of L-ornithine gives rise to that proton at C-1 of putrescine which is lost, that is, the *pro*-*S* proton. It follows that the decarboxylation of ornithine to putrescine in intact *N. tabacum* takes place with retention of configuration. The decarboxylation of ornithine, catalysed by ornithine decarboxylase of bacterial origin, likewise takes place with retention of configuration.[10]

The stereochemistry of the final step in nicotine biosynthesis follows from the chirality of the product, (*S*)-nicotine (**7**). Attack by the nicotinic acid-derived fragment (**6**) on the *N*-methyl-1-pyrrolinium ion (**5**) occurs from the 1-*si*,2-*re* face of the latter.

The four stereochemical ambiguities of nicotine biosynthesis are thus resolved.

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

Received, 11th February 1982; Com. 147

References

1 L. J. Dewey, R. U. Byerrum, and C. D. Ball, *Biochim. Biophys. Acta*, 1955, **18**, 141.
2 E. Leete, *Chem. Ind. (London)*, 1955, 537.
3 E. Leete, *J. Am. Chem. Soc.*, 1967, **89**, 7081.
4 E. Leete, in 'Biosynthesis,' ed. J. D. Bu'Lock (Specialist Periodical Reports), The Chemical Society, London, 1976, vol. 4, p. 97.
5 E. Leete, *J. Am. Chem. Soc.*, 1958, **80**, 2162.
6 E. Leete and J. A. McDonell, *J. Am. Chem. Soc.*, 1981, **103**, 658.
7 T. Kisaki, S. Mizusaki, and E. Tamaki, *Arch. Biochem. Biophys.*, 1966, **117**, 677.
8 S. Mizusaki, Y. Tanabe, M. Noguchi, and E. Tamaki, *Plant Cell Physiol.*, 1971, **12**, 633.
9 S. Mizusaki, Y. Tanabe, M. Noguchi, and E. Tamaki, *Phytochemistry*, 1972, **11**, 2757.
10 J. C. Richards and I. D. Spenser, reported at the 62nd Canadian Chemical Conference, C.I.C., Vancouver 1979, Abstract No. OR-120, to be submitted for publication.
11 T. P. Pitner, W. B. Edwards, III, R. L. Bassfield, and J. F. Whidby, *J. Am. Chem. Soc.*, 1978, **100**, 246.
12 A. R. Battersby, J. Staunton, and M. C. Summers, *J. Chem. Soc., Perkin Trans.* 1, 1976, 1052.
13 J. C. Richards and I. D. Spenser, *J. Am. Chem. Soc.*, 1978, **100**, 7402.
14 H. J. Gerdes and E. Leistner, *Phytochemistry*, 1979, **18**, 771.
15 E. Leistner, R. N. Gupta, and I. D. Spenser, *J. Am. Chem. Soc.*, 1973, **95**, 4040.
16 L. J. J. Mestichelli, R. N. Gupta, and I. D. Spenser, *J. Biol. Chem.*, 1979, **254**, 640.