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THE INCORPORATION OF ORNITHINE-[2,3-$^{13}C_2$] INTO NICOTINE AND NORNICOTINE ESTABLISHED BY NMR

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Key Word Index—*Nicotiana glutinosa*; Solanaceae; nicotine; nornicotine; ornithine; ^{13}C NMR spectroscopy; biosynthesis.

Abstract—DL-Ornithine-[2,3-$^{13}C_2$] was synthesized from acetate-[1-^{13}C] and ethyl acetamidocyanoacetate-[2-^{13}C]. This labelled material was mixed with DL-ornithine-[5-^{14}C] and fed to *Nicotiana glutinosa* plants by the wick method. After 10 days the plants were harvested affording radioactive nicotine and nornicotine (0.14% and 0.051% specific incorporations, respectively). Even at these low specific incorporations an examination of their ^{13}C NMR spectra established the incorporation of ornithine symmetrically into the pyrrolidine rings of these alkaloids. Satellites were observable at the signals due to C-2′, 3′, 4′ and 5′ positions, arising by the presence of contiguous carbons at C-2′, 3′ and C-4′, 5′.

INTRODUCTION

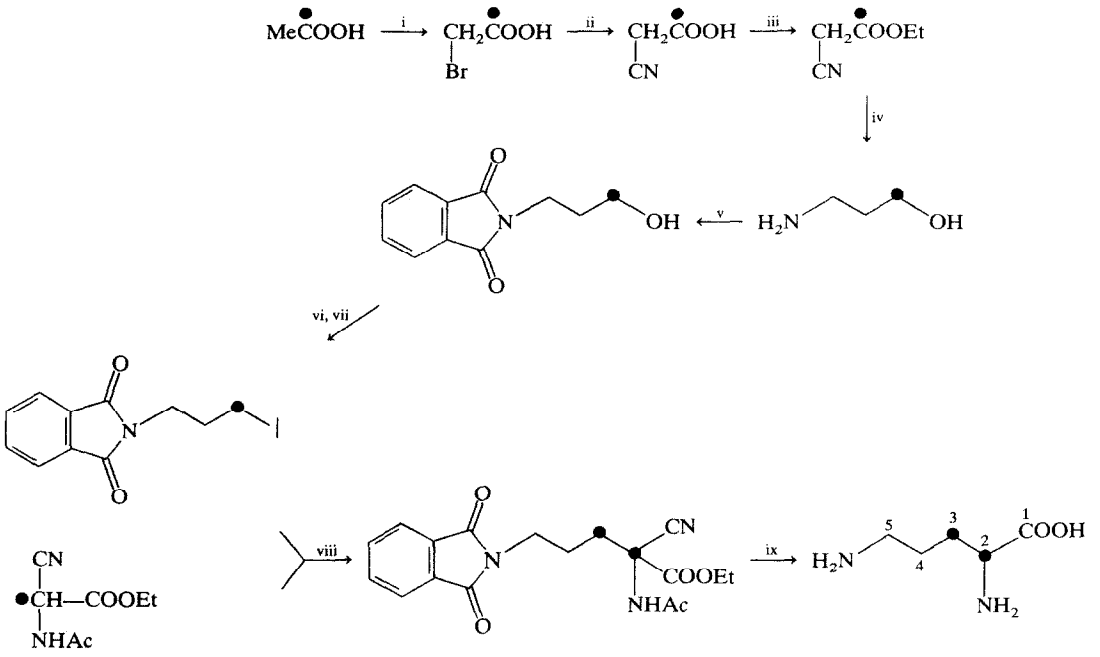
It has been previously [1] established that the administration of radioactive ornithine (labelled at C-2 or C-5) to *Nicotiana* species results in the formation of nicotine and nornicotine which are labelled equally at C-2′ and C-5′ of their pyrrolidine rings. This result has been rationalized [2] by proposing that ornithine is incorporated into nicotine via putrescine, *N*-methylputrescine and an *N*-methyl-Δ^1-pyrrolinium salt. The formation of nicotine symmetrically labelled at C-2′ and C-5′ from ornithine-[2-^{14}C] is thus due to the intermediacy of free putrescine, a symmetrical compound. Work with enzymes [3] isolated from tobacco roots support this proposed biosynthetic pathway. However, the work of Rapoport and co-workers [4] which involved the growing of tobacco in $^{14}CO_2$ is not compatible with this pathway since unsymmetrical labelling of the pyrrolidine ring was, on occasion, observed. This work has been reviewed [5, 6] and it was hoped that the administration of $^{13}CO_2$ to tobacco with subsequent determination of the location of the ^{13}C in the labelled nicotine by ^{13}C NMR, would resolve this paradox. Unfortunately, the work with $^{13}C_2$ was equivocal [7], it being suggested in the most recent publication of Hutchinson [8] that $^{13}CO_2$ did yield nicotine which was unsymmetrically labelled, by an analysis of its ^{13}C NMR spectrum. In the present work we have tested the ^{13}C NMR method of determining the distribution of the ^{13}C isotope in the pyrrolidine rings of nicotine and nornicotine by feeding ornithine-[2,3-$^{13}C_2$] to tobacco. The expectation was that the pyrrolidine rings would be labelled symmetrically at C-2′, 3′ and C-4′, 5′ resulting in the formation of satellites at these carbons due to spin–spin coupling of the contiguous ^{13}C atoms.

* Contribution No. 167 from this laboratory.

RESULTS AND DISCUSSION

The ornithine-[2,3-$^{13}C_2$] was prepared by the route illustrated in Scheme 1 using the indicated reagents. The sequence of reactions is based on known transformations and the overall yield from acetic acid-[1-^{13}C] was only 1.5%, however all the steps have not been optimized. The distribution of ^{13}C (doubly and singly labelled species) in the ornithine was deduced from the ^1H and ^{13}C NMR spectra of intermediates in this sequence (see Experimental). The ^{13}C NMR spectrum of the ^{13}C-labelled ornithine hydrochloride (in D_2O) is illustrated in Fig. 1. The highly enriched carbons 2 and 3 are observed as triplets, the central peaks arising from singly labelled species (ornithine-[2-^{13}C] and ornithine-[3-^{13}C]) the satellites being due to the ornithine-[2,3-$^{13}C_2$]. The natural abundance carbons, made visible by attenuating the spectrum ×25, were also split into triplets by coupling with the highly enriched carbons. It should be noted that the satellites are not symmetrically located about the central peaks (see Table 1). This is especially noticeable when the coupled carbons have chemical shifts which are close together (e.g. C-3 and C-4), and is due an approach to an AB spin system [9].

This ^{13}C-labelled ornithine was mixed with a small amount of ornithine-[5-^{14}C] in order to facilitate determination of its specific incorporation into the alkaloids by means of radioactive assay. Administration to *N. glutinosa* plants was carried out by the wick-feeding method. After 10 days the whole plants were extracted as previously described [10] affording a mixture of nicotine and nornicotine. Even though the specific incorporation (^{14}C) of the ornithine-[5-^{14}C, 2,3-$^{13}C_2$] into nicotine was low (0.14%) an examination of its ^{13}C NMR spectrum (Fig. 2) reveals the presence of satellites at C-2′, 3′, 4′ and 5′. Fig. 3



Scheme 1. Synthesis of ornithine-[2,3-$^{13}C_2$]. The labelled atoms are indicated with heavy dots. i, Br_2/P; ii, $NaHCO_3$, NaCN; iii, $EtOH/H^+$; iv, $LiAlH_4$; v, Phthalic anhydride; vi, PBr_3; vii, NaI in Me_2CO; viii, NaOEt; ix, conc HCl.

illustrates the signal for C-5′ expanded in the horizontal and vertical scales. From the intensities of the satellite and central peaks (determined by cutting out the expanded spectra and weighing) it is possible to calculate [11] the specific incorporation of the ornithine-[2,3-$^{13}C_2$] into each carbon of the pyrrolidine ring. The calculations are outlined in Fig. 3. In nicotine the average specific incorporation at C-2′ and C-3′ was 0.077%, and at C-4′ and C-5′, it was 0.077%. The incorporation of the labelled ornithine into nornicotine was even lower (0.051%). This was to be expected since nornicotine is a metabolite of nicotine [12]. However, satellites were observable in the ^{13}C NMR spectrum of this nornicotine at C-2′, 3′, 4′ and 5′, from which the specific incorporations could be calculated (see Experimental). With these low incorporations it is important to subtract from the observed satellite peaks the contribution resulting from the natural abundance of contiguous ^{13}C atom (0.0123%). Here also the incorporation of ^{13}C into the two sides of the pyrrolidine ring was almost identical (0.026 and 0.031%). In both nicotine and nornicotine the calculated incorporation of ^{13}C into C-2′ was significantly less than the incorporations into C-3′, 4′ and 5′. We do not presently understand this observation but it could be related to the fact that this carbon is directly attached to the pyridine ring.

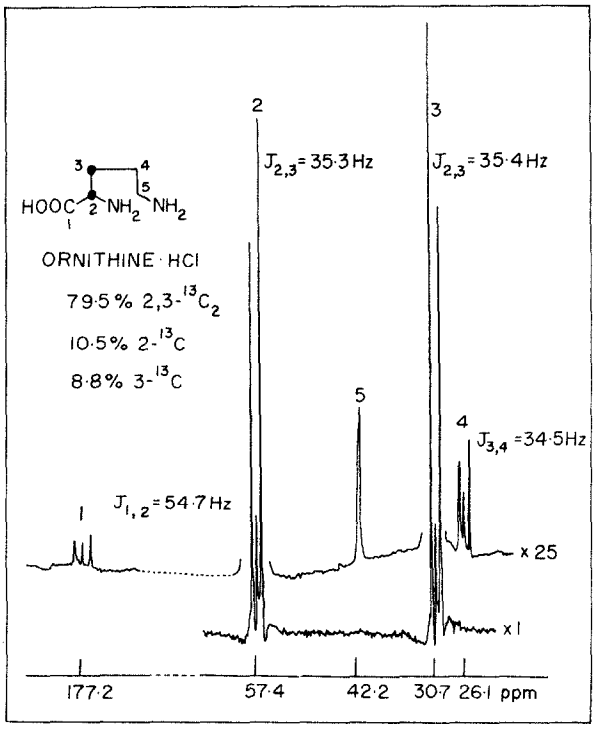


Fig. 1. ^{13}C NMR spectrum of ornithine-[2,3-$^{13}C_2$] hydrochloride (in D_2O).

We consider that the present results completely validate the previous work on the incorporation of ornithine into the pyrrolidine ring of the tobacco alkaloids. It also demonstrates that precursors labelled with contiguous ^{13}C atoms are especially useful for determining biosynthetic pathways since the satellites which arise from such precursors can be detected at high dilutions (*ca* 4000).

EXPERIMENTAL

General methods. Radioactive materials were assayed in duplicate in a liquid scintillation counter using dioxane–EtOH with the usual scintillators [13]. ^{13}C NMR spectra

Table 1. Coupling constants for ornithine-[2,3-$^{13}C_2$] and the alkaloids derived from it

Compound	Coupled carbons	V^*_{AB} (Hz)	J_{AB} (Hz)	Distance between central peak and inner satellite (Hz)	Calculated distance between central peak and inner satellite†
Ornithine-[2,3-$^{13}C_2$]	1–2	3019.4	54.7	27.4	27.4
Nicotine	2–3	672.1	35.3	17.1	17.2
			35.4	16.9	
	3–4	118.5	34.5	15.2	14.8
	2′–3′	845.0	35.0	17.5	17.1
			34.7	16.8	
	4′–5′	863.0	34.4	16.7	16.8
			34.3	16.8	
Nornicotine	2′–3′	642.5	33.1	16.2	16.2
			33.6	16.2	
	4′–5′	538.6	32.7	15.8	15.9
			32.8	16.0	

* V_{AB} = difference in chemical shift between the coupled carbons.

† Calculated from the formula: $\frac{V_{AB}+J_{AB}-\sqrt{V^2_{AB}+J^2_{AB}}}{2}$.

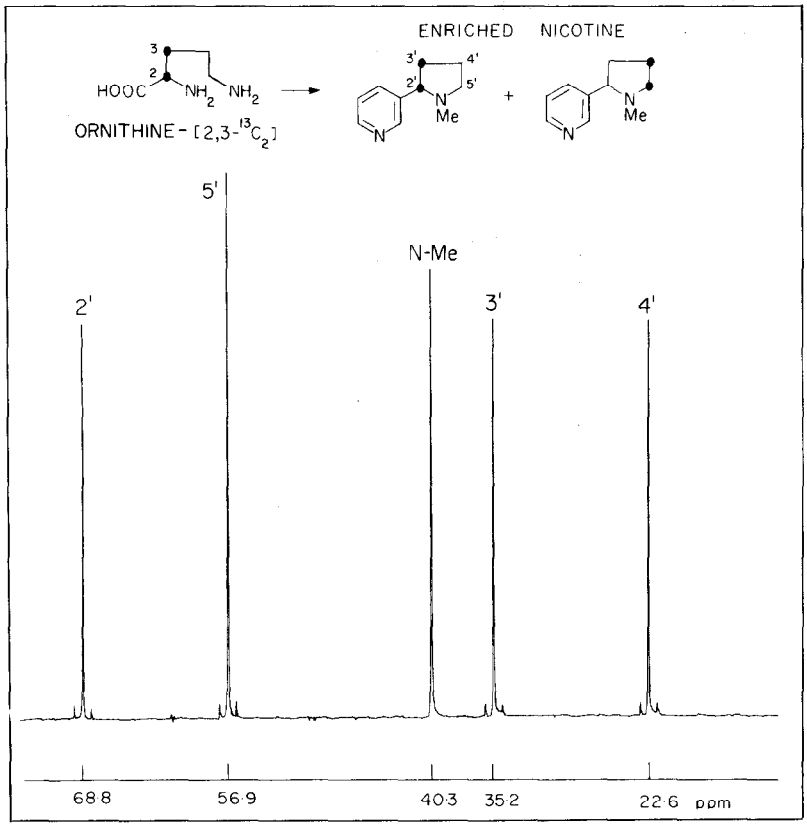


Fig. 2. ^{13}C NMR spectrum of enriched nicotine (aliphatic region) derived from ornithine-[2,3-$^{13}C_2$] (chemical shifts ppm from TMS).

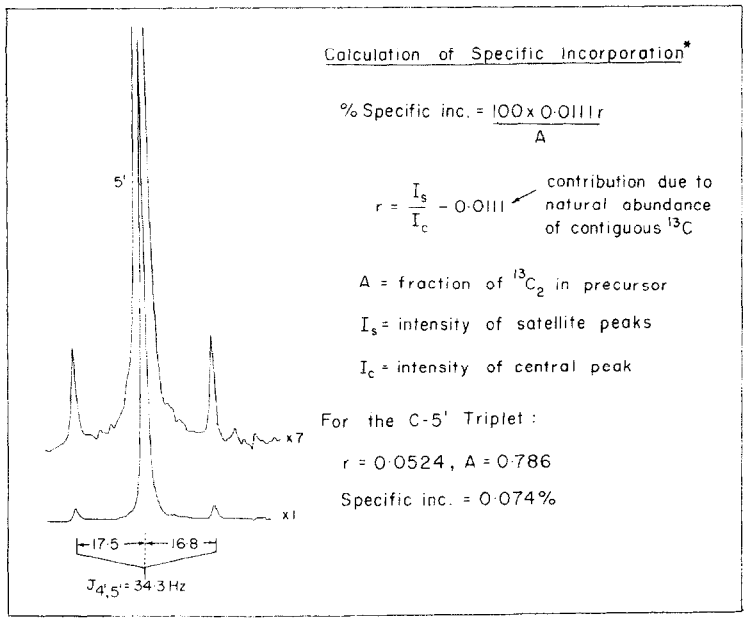


Fig. 3. ^{13}C NMR spectrum of C-5' of nicotine, illustrating method of determining specific incorporation.

were obtained at 25.2 MHz using an instrument equipped with Fourier transform accessory. ^1H NMR spectra were obtained at 80 MHz.

DL-*Ornithine*-[2,3-$^{13}C_2$]. Acetic acid-[1-^{13}C] (8.5 g, 0.139 M) was converted to ethyl cyanoacetate-[1-^{13}C] (11.2 g 70.5%) via bromoacetic acid-[1-^{13}C] [14, 15] and cyanoacetic acid-[1-^{13}C] [16]. This ester dissolved in Et_2O (110 ml) was added slowly (over 45 min) to a soln of $LiAlH_4$ (17.6 g, 0.44 M) in Et_2O (1 l.) at −78° in a N_2 atmosphere. The mixture was then stirred at room temp. for an additional 2 hr. H_2O (52 ml) was then slowly added and the mixture stirred for 2 days. The residue obtained on filtration was stirred with additional quantities of Et_2O (2 × 1 l.) also for 2 days. The combined Et_2O filtrates were dried (Na_2SO_4) and evapd affording 3-aminopropanol-[1-^{13}C] (2.22 g, 29.2 mM, 29.7%) [17]. Finely-powdered phthalic anhydride (6.59 g, 44 mM) was added to this amine in a 100 ml flask fitted with a dropping funnel and a $CaCl_2$ drying tube. After the spontaneous reaction had ceased, freshly dist PBr_3 (2.78 ml) was slowly added and the mixture heated at 85–90° for 1 hr. The cooled reaction mixture was then added to ice and extracted with Et_2O (3 × 60 ml). The Et_2O extract was washed with 5% Na_2CO_3 to remove phthalic acid and HBr. The residue obtained on evapn of the dried (Na_2CO_3) extract was crystallized from EtOH affording 3-bromopropylphthalimide-[3-^{13}C] (1.92 g, 24.7%), mp 73–74° (lit. 73–75° [18]); ^1H NMR ($CDCl_3$): δ 7.74 (4 aromatic H, *m*), 3.81 (N-CH_2, *m*), 2.31 (N-CH_2CH_2, *m*), three triplets at 4.34, 3.44, 2.38 arising from the C-3 hydrogens which are split by ^{13}C ($J_{^{13}C-H}$ = 159 Hz). From the intensity of the triplet at 4.34 ($^{13}CH_2$) to that of the one at 3.44 ($^{12}CH_2$) it was calculated that the ^{13}C enrichment at C-3 was 88.3%. The 3-bromopropylphthalimide-[3-^{13}C] (1.83 g, 6.83 mM) and NaI (7.9 g, 52.7 mM) were dissolved in Me_2CO (50 ml) and the mixture refluxed for 18 hr. The residue obtained on evapn of the solvent was washed with H_2O affording 3-iodopropylphthalimide-[3-^{13}C] (2.02 g, 94%), mp 87–88° (lit. 88–89° [19]). This material (1.87 g, 5.92 mM) was condensed with ethyl acetamidocyanocetate-[2-^{13}C] 90% ^{13}C (purchased from Merck or synthesized from acetic acid-[2-^{13}C] by established procedures [19]) (1.01 g, 5.91 mM) with NaOEt in EtOH [19] to afford ethyl-2-cyano-2-acetamido-5-phthalimidopentanoate-[2,3-$^{13}C_2$] (0.993 g, 46.7%) which was hydrolysed with 11 M HCl [19] to yield DL-ornithine-[2,3-$^{13}C_2$] isolated as its mono HCl (0.304 g, 64%) mp 225–228° (lit. 225° [18]). Its ^{13}C NMR spectra (in D_2O) along with the chemical shifts (relative to TMS) are recorded in Fig. 1. The coupling constants are recorded in Table 1.

Administration of DL-*ornithine*-[5-^{14}C, 2,3,-$^{13}C_2$] *to* Nicotiana glutinosa *plants, and isolation of alkaloids.* The DL-ornithine-[2,3-$^{13}C_2$] (HCl) was diluted with some DL-ornithine-[5-^{14}C] (Research Products International,

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* The formula below can be used when the specific incorporation is low (<0.2%). When higher incorporations are observed the contribution of singly labelled precursors, e.g. in this case the ornithine-[2-$^{13}C_1$] to the central and satellite peaks must be taken into account. The specific incorporation can then be calculated from the formula:

$$100 \times \frac{0.0111\,r}{A\left(\frac{I_s}{I_{c'}}\right) - r(B - 0.0111)}$$

where

$$r = \frac{I_s}{I_{c'}} - 0.111$$

and

$$I_{c'} = \frac{I_c\left(1 - \frac{I_s}{I_c} \cdot \frac{(B - 0.0111)}{A}\right)}{1 - 0.0111\left(\frac{B - 0.0111}{A}\right)},$$

where I_s and I_c are the measured intensities of the satellites and central peaks, respectively, A = fraction of the $^{13}C_2$ species, B = fraction of the $^{13}C_1$ species.

11.5 mCi/mM) to afford material (25.38 mg), having a sp. act. (^{14}C) of 2.43×10^8 dpm/mM, and a ^{13}C distribution as follows: [2, 3-$^{13}C_2$] 78.6%, [2-^{13}C] 10.4%, [3-^{13}C] 8.7%. The plants (6) were 4 months old growing in soil in a greenhouse and were fed (in December) by means of cotton wicks inserted into the stems of the plants 20 cm above the soil level. After 10 days the plants were harvested (residual activity in the beakers supplying the cotton wicks: 0.03%). The plants (fr. wt: 600 g) yielded nicotine (151 mg, 3.40×10^5 dpm/mM, 0.14% sp. incorpn) and nornicotine (225 mg, 1.24×10^5 dpm/mM, 0.051% sp. incorpn).

Determination of ^{13}C NMR spectra of nicotine and nornicotine. The spectra recorded in Figs. 2 and 3 were carried out on the enriched nicotine (85 mg in 0.4 ml $CDCl_3$) in a 5 mm tube, 47 K transients, 1.43 sec acquisition time; 0.7 Hz data point, a sweep width of 2800 Hz and a pulse width of 135 μsec. The sp. incorpns, calculated (as illustrated Fig. 3) from the intensities of the satellites, compared with the central peaks were: C-2′: 0.066, C-3′: 0.089, C-4′: 0.081, C-5′: 0.074%. The ^{13}C NMR on nornicotine (180 mg in 0.3 ml $CDCl_3$) was determined with identical instrument parameters (54 K transients). The sp. incorpns deduced from the spectra were: C-2′: 0.020, C-3′: 0.032, C-4′: 0.030, C-5′: 0.032%. The coupling constants of the coupled carbons are recorded in Table 1.

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