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BIOSYNTHESIS OF NORNICOTINE IN ROOT CULTURES OF *NICOTIANA ALATA* DOES NOT INVOLVE OXIDATION AT C-5′ OF NICOTINE

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Key Word Index—*Nicotiana alata*; solanaceae; alkaloid biosynthesis; demethylation; nicotine; root cultures.

Abstract—The mechanism of the demethylation of nicotine [3-(1-methyl-2-pyrrolidinyl)pyridine] to nornicotine [3-(2-pyrrolidinyl)pyridine] has been studied in root cultures of *Nicotiana alata* using nicotine selectively labelled with ^2H. Administered [4′,4′,5′,5′-$^2H_4$]nicotine was effectively incorporated into [4′,4′,5′,5′-$^2H_4$]nornicotine without a significant alteration in the molar fractions of ^2H at the four substituted positions, as determined by quantitative ^2H NMR. Incorporation into cotinine-d_2 and myosmine-d_4 was also observed. In contrast, no incorporation into either nornicotine or nicotine was found when [4′,4′-$^2H_2$]cotinine was administered. Taken together, these findings provide direct evidence that neither of the protons on C-5′ of nicotine takes part in the demethylation reaction.

INTRODUCTION

(*S*)-Nicotine is the principal pyrrolidine alkaloid found in the genus *Nicotiana* [1]. The 1-methylpyrrolidine ring of nicotine is derived (Fig. 1) from the amino acid L-arginine *via* putrescine and *N*-methylputrescine [2]. Deaminative oxidation of *N*-methylputrescine [3] yields *N*-methylaminobutanal, which undergoes spontaneous ring-closure in mild conditions to form a *N*-methylpyrrolinium ion. The pyridine moiety is derived from nicotinic acid and is introduced by a poorly-understood oxygen-dependent decarboxylative condensation [4, 5].

In addition to nicotine, a range of pyrrolidine and piperidine alkaloids are known from fresh *Nicotiana* tissue [1, 6]. In some species, these may be more prevalent than nicotine [1]. The piperidine alkaloids are derived from L-lysine by an analogous route [7]. A number of the pyrrolidine alkaloids present—notably nornicotine, cotinine and myosmine—appear to be oxidative metabolites of nicotine, rather than the products of parallel pathways from analogous pyrrolidine precursors [6]. While it has been acknowledged for some time that nornicotine in the living plant is derived by the demethylation of nicotine [6, 8], the mechanism of this process is not understood.

Nicotine oxidation by microorganisms and by mammals has been extensively studied [8, 9]. With the fungus *Cunninghamella echinulata*, nornicotine and *N*-methylmyosmine are the principal products, albeit in low yields [10]. Hepatic homogenates, in contrast, produce cotinine as the major and nornicotine as the minor products from nicotine (Fig. 2) [9, 11]. In this latter case, there is strong evidence for the involvement of iminium intermediates [12].

A number of mechanisms for the demethylation of nicotine in plants have been proposed [6, 8] involving

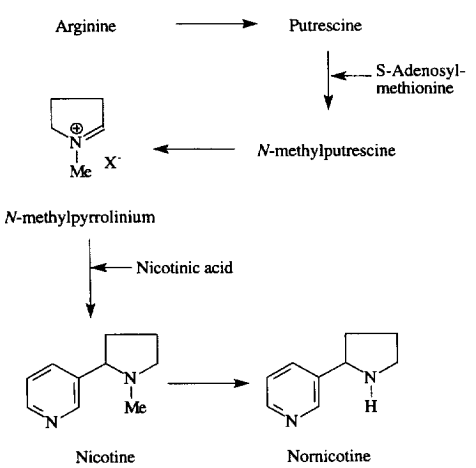

Fig. 1. Abbreviated biosynthetic pathway for the formation of nicotine and nornicotine from arginine, *S*-adenosylmethionine and nicotinic acid.

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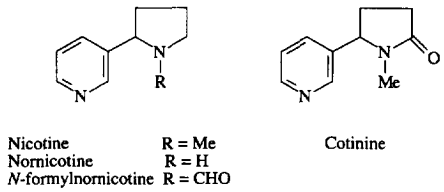
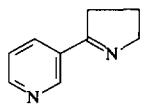
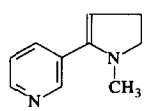


Nicotine R = Me
Nornicotine R = H
N-formylnornicotine R = CHO

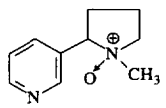
Cotinine



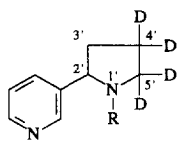
Myosmine



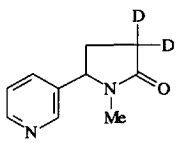
N-Methylmyosmine



Nicotine-1′-*N*-oxide



[4′,4′,5′,5′-$^2H_4$]Nicotine R = Me
[4′,4′,5′,5′-$^2H_4$]Nornicotine R = H



[4′,4′-$^2H_2$]Cotinine

oxidation at the *N*-methyl group, oxidation at the C-2′ position or oxidation at the C-5′ position. Several investigations have concluded that nicotine-1′-*N*-oxide is probably not an intermediate [6, 8], while oxidation (hydrogen loss) at C-2′ is effectively eliminated, because the ^3H-label was retained in isolated nornicotine when (*S*)-[2′-^{14}C,2′-^{3}H]nicotine was fed to *N. glauca* plants [13]. Thus, myosmine and *N*-methylmyosmine are excluded as intermediates. Furthermore, there is preliminary evidence that demethylation involves a heme-thiolate P_{450} and has a requirement both for activated molecular oxygen [14] and NADPH [15]. As, on the basis of the evidence cited above, the product of this activity appears not to be a *N*-oxide, the oxygen atom must be introduced at either the methyl or the C-5′ position.

By analogy with the mammalian oxidative pathway (Fig. 2), it is possible that an intermediate oxidised at C-5′ might also be involved in plants. This could be due either to the loss of one proton, as in 5′-hydroxynicotine, or to complete oxidation at C-5′ with the loss of two protons, as in cotinine. In order to test any such involvement, [4′,4′,5′,5′-$^2H_4$]nicotine was prepared, fed to root cultures of *N. alata* and the labelling patterns in the oxidation products examined by quantitative ^2H NMR and GC-mass spectroscopy. Root and suspension cultures of various *Nicotiana* spp. have previously proved to be efficient at converting added nicotine to nornicotine [16–18].

RESULTS AND DISCUSSION

Root cultures of *N. alata*, propagated as described in the Experimental section, produce a profile of alkaloids similar to that found in the roots of intact plants [1], with nornicotine, anatabine and nicotine as the major components and trace amounts of anabasine, myosmine and a number of unidentified alkaloids. Exogenously supplied nicotine is efficiently converted to nornicotine and cotinine.

To begin the biosynthetic study, [4′,4′,5′,5′-$^2H_4$] nic-

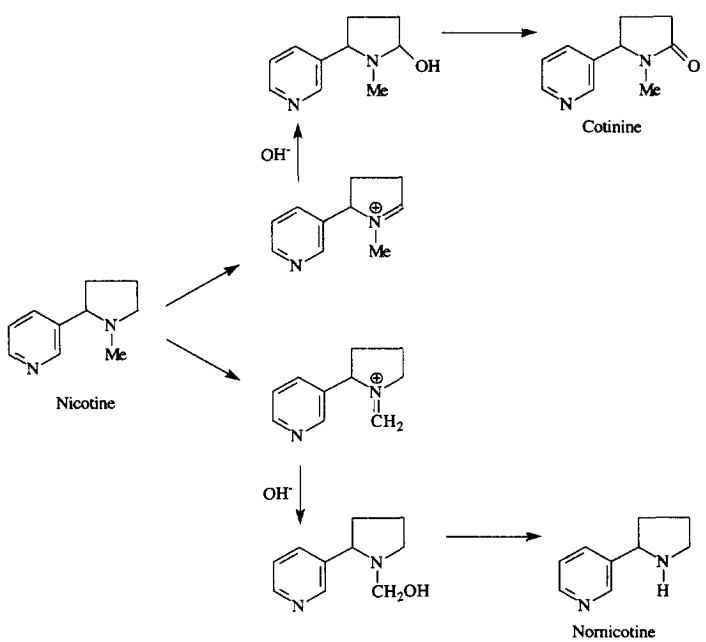


Fig. 2. Oxidation of nicotine to cotinine and nornicotine *via* iminium intermediates by hepatic microsomes [11, 12].

Table 1. Molar fractions of ^2H in [4′,4′,5′,5′-$^2H_4$]nicotine and [4′,4′,5′,5′-$^2H_4$]nornicotine derived therefrom as determined by quantitative ^2H NMR. Each determination was performed N times and is expressed ± s.d.

Material	Mass (mg)	N	Position* 4′a	4′b	5′a	5′b
Displacement (ppm)			1.89	1.98	2.46	3.30
[4′,4′,5′,5′-$^2H_4$]Nicotine	7.0	4	0.2416 ± 0.0004	0.2352 ± 0.0013	0.2632 ± 0.0009	0.2600 ± 0.0006
[4′,4′,5′,5′-$^2H_4$]Nicotine	1.4	3	0.2374 ± 0.0032	0.2326 ± 0.0017	0.2700 ± 0.0040	0.2600 ± 0.0008
Displacement (ppm)			1.84	1.92	3.16	3.24
[4′,4′,5′,5′-$^2H_4$]Nornicotine	c.5	4	0.2695 ± 0.0014	0.2410 ± 0.0012	0.2525 ± 0.0007	0.2371 ± 0.0018

*See Fig. 1. Assignments used as determined in ref. [23]. The proton H-5′a (axial) is that *trans* to the lone pair on the nitrogen atom.

otine (83% $^2H_4$ as determined by GC-mass spectrometry) was prepared from nicotine (see Experimental). The incorporation of ^2H into the 4′,4′ and 5′,5′ positions was measured precisely on an aliquot using quantitative ^2H NMR [Table 1; Fig. 3(a)]. The enriched nicotine was fed at 0.6 mM (0.03 mmol) to three-day-old root cultures of *N. alata* and the crude alkaloid mixture extracted five days later and analysed by GC-mass spectrometry. The feeding was repeated twice. A number of alkaloids could be identified in this extract, the principal ones of which were nornicotine (*ca* 58%), nicotine (*ca* 8%), cotinine (*ca* 16%) and anatabine (*ca* 13%). A third feed was made at 1.2 mM (0.06 mmol) and the alkaloids extracted after 10 days; this was richer in nornicotine (*ca* 70%). The extracted alkaloids were pooled for ^2H NMR analysis. Incorporation of ^2H-label into these bases was determined by GC-mass spectrometry (Table 2). The extent of labelling in the recovered alkaloids was variable, depending on the length of time for which the cultures were left.

Recovered nornicotine, cotinine and myosmine (data not shown) all showed high incorporations, in the same range as the added nicotine (Table 2). The recovered [4′,4′-$^2H_2$]cotinine had a specific incorporation equivalent to that of the 4′,4′-$^2H_2$ of the fed nicotine (values in parentheses, Table 2), indicating that this metabolite was entirely due to the oxidation of the exhibited nicotine. Only traces of cotinine were detected in unfed controls. In contrast to previous authors working with whole plants [13], we found that fed labelled nicotine was converted to cotinine, with yields between 5 and 10%. This probably reflects the much more efficient incorporation normally achieved with root cultures [16]. The conversion of labelled nicotine to nornicotine was, however, much more significant (between 70 and 90%).

As expected, no incorporation of [4′,4′,5′,5′-$^2H_4$]nicotine into anatabine or anabasine was found (data not shown), reflecting the established different biosynthetic origins of these piperidine bases [6].

The crude alkaloid mixture was subjected to preparative silica gel TLC. Nornicotine was isolated ($R_f \approx 0.4$), recovered by eluting the silica gel with MeOH–32% NH_4OH (19:1) and the purity established by GC-mass spectrometry. Analysis of this material by ^1H NMR confirmed the structure to be nornicotine. From the ^2H NMR spectrum [Table 1; Fig. 3(b)] it is clear that all four ^2H atoms present in the exhibited nicotine had been retained in the product without significant change in their molar ratios.

In order to confirm that cotinine is not metabolised to nornicotine, either directly or *via* a route involving prior reduction to nicotine, [4′,4′-$^2H_2$]cotinine (92% $^2H_2$ as determined by GC-mass spectrometry), was fed at 0.6 mM to 3-day-old root cultures of *N. alata* and the alkaloids extracted after a further 5 days. Although the recovered cotinine was still fully enriched (98%), no enrichment of nicotine or nornicotine was observed (Table 2). Furthermore, no norcotinine, the demethylation product of this substrate, was detected by GC-mass spectrometry.

CONCLUSIONS

It can be concluded, therefore, that the demethylation of nicotine to nornicotine does not involve a reaction in which any one of the 4′,4′ or 5′,5′ protons is displaced. If this were to happen, the enrichment at the relevant position would have been significantly altered in relation to that recovered at the unaffected positions. This would have been readily observed in the ^2H NMR spectrum of nornicotine. In contrast, these data prove that all four protons remain in place during the demethylation of nicotine. Since the involvement of the 2′-H has previously been eliminated [6], it appears most probable that the oxidative demethylation takes place as a result of the oxidation of the *N*-methyl group. This may involve dehydrogenation to the methyleneiminium species and the addition of water, as described for hepatic microsomes [11, 12] (Fig. 2), or direct oxidation to *N*-formylnornicotine and *N*-hydroxymethylnornicotine. *N*-Formylnornicotine is identified as a minor metabolite during the demethylation of nicotine by *N. tabacum* suspension cultures [18], is reported from gland exudates of tobacco leaves [20] and is a minor oxidation product found during curing [6]. The oxidative

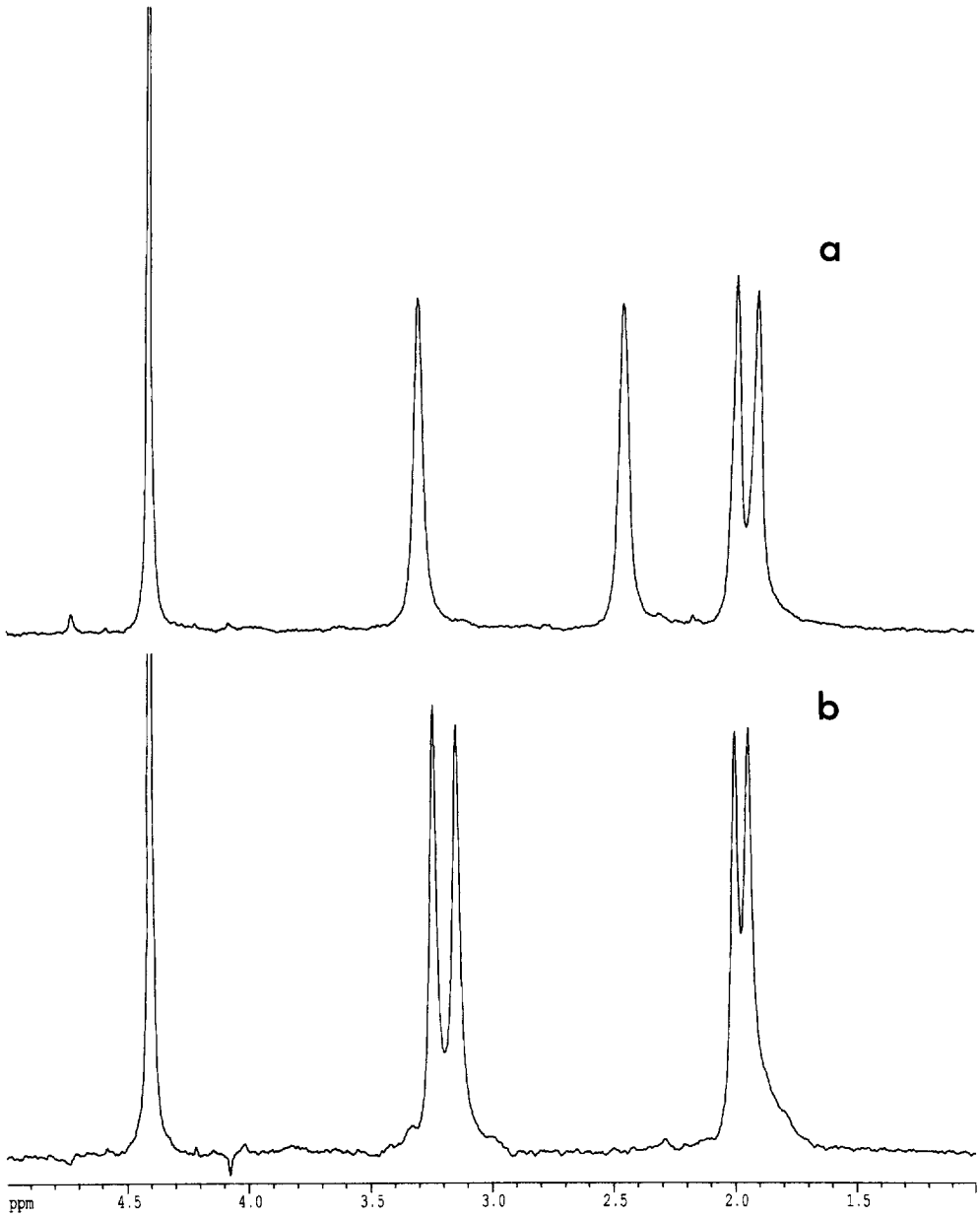


Fig. 3. 61.4 MHz ^{2}H NMR spectra of (a) [4′,4′,5′,5′-$^2H_4$]nicotine and (b) [4′,4′,5′,5′-$^2H_4$]nornicotine with nitromethane as internal reference at δ 4.40.

reaction may, however, be concerted, there normally being little free *N*-formylnornicotine produced. This would be compatible with the preliminary evidence that the reaction involves a hemethiolate P_{450} activity [14, 15], in which complete oxidation would occur without release of intermediates.

EXPERIMENTAL

General. ^{1}H NMR were recorded at 500 MHz, ^{2}H NMR at 61.4 MHz. Quantification of peak areas was achieved using INTERLISS (© Eurofins S.A., Nantes, France). Spectra were collected in at least triplicate.

Cultures. Sterile shoot cultures of *N. alata* were obtained from the Royal Botanic Gardens, Kew, U.K. Root cultures were established by transferring the excised roots of 4 to 8-week-old shoots to liquid half-strength Murashige and Skoog medium (ICN Inc.), supplemented with 15 g l^{-1} sucrose (50 ml per 250 ml Erlenmeyer flask), essentially as described previously [21]. Cultures were maintained at 25° with gyratory shaking at 90 rpm and constant low illumination. They were passaged into fresh medium every 7 days.

Table 2. Specific incorporations of [4′,4′,5′,5′-$^2H_4$]nicotine and [4′,4′-$^2H_2$]cotinine into alkaloids in root cultures of *N. alata*, as determined by GC-mass spectrometry

Alkaloid fed	Mass fed (mmol flask^{-1})	Period (days)	Status	Specific Incorporation (%)*† at M+4 into Nicotine	Nornicotine	Cotinine
[4′,4′,5′,5′-$^2H_4$]Nicotine	0.03	5	Crude	83.7	81.8	105.8 (97.2)‡
[4′,4′,5′,5′-$^2H_4$]Nicotine	0.06§	10	Crude	47.1	56.3	108.3 (98.1)‡
			Fractions	42.4	59.2	n.d.¶
[4′,4′-$^2H_2$]Cotinine	0.03	5	Crude	<0.1	<0.1	98.2
			Fractions	0.5	1.4	n.d.

* Specific Incorporation (%) = 100 × (excess of isotope in isolated product/excess of isotope in precursor).

† EI-induced fragmentation of nicotine and related alkaloids leads to a major ion at $[M-1]^+$ [22]. All ion intensities have therefore been corrected to take into account the contribution due to the species at −1 mu.

‡ Specific incorporation >100% in cotinine reflects the fact that the 4′,4′-positions of the fed nicotine-d_4 are more enriched (92% as measured by GC-MS) than the 5′,5′ positions (83% as measured by GC-MS), due to the synthetic route used (see Experimental). The value in parentheses gives the specific enrichment in relation to the 4′,4′-positions of nicotine only.

§ Fed as 0.03 mmol on day 3 and 0.03 mmol on day 7.

¶ n.d. = not determined.

Substances to be fed were dissolved at 12 mM in H_2O as the HCl salt, between pH 5 and 7 and filter-sterilised directly into the flask (2.5 ml, giving 0.6 mM final concn). Typically, roots were fed at 3 days-old and harvested 4 to 7 days later.

Precursors. [4′,4′,5′,5′-$^2H_4$]Nicotine was synthesized from (*S*)-(−)-nicotine (Fluka Chemika) by a modification of published procedures [22, 23].

4′,4′-*Dibromocotinine.* To nicotine (5 g, 31 mmol) dissolved in HOAc–H_2O (4:1, 15 ml) was added Br_2 (18 g, 112 mmol, dissolved in 18 ml of the same solvent), dropwise over 1 hr with continuous stirring at room temp. After a further 2 hr, the two phases were mixed by warming to 60° with rapid agitation and the soln left to cool slowly. After 2 to 5 days, orange crystals were recovered, washed with a little H_2O, dried *in vacuo*, and confirmed [24] to be of 4′,4′-dibromocotinine. HBr; on the free base δ_H (500 MHz; $CDCl_3$) 2.94 (H, *dd*, $J_{2'\text{-}3'a}$ = 8 Hz, $J_{3'a\text{-}3'b}$ = 14.7 Hz, H-3′a), 3.52 (H, *dd*, $J_{2'\text{-}3'b}$ = 6 Hz, $J_{3'a\text{-}3'b}$ = 14.7 Hz, H-3′b), 4.64 (H, *dd*, $J_{2'\text{-}3'a}$ = 8 Hz, $J_{2'\text{-}3'b}$ = 6 Hz, H-2′); δ_C (125 MHz; $CDCl_3$) 39.21 (Me), 53.13 (C-3′), 59.48 (C-4′), 69.04 (C-2′), 168.15 (C-5′).

[4′,4′-$^2H_2$]*Cotinine.* To 4′,4′-dibromocotinine. HBr (5 g, 21 mmol) dissolved in HOAc (7.5 ml) and ^2H_2O (20 g, 1 mol) retained under N_2 gas, was added over 20 min at room temp. and with rapid stirring, freshly washed Zn granules (5 g, 76 mmol). After 1 hr, when the soln showed no further decolorisation, excess Zn was removed by filtration, the soln basified and the alkaloids extracted with $CHCl_3$. On removal of solvent *in vacuo*, a pale brown oleaginous residue was obtained which was confirmed [22, 24] to be [4′,4′-$^2H_2$]cotinine; δ_H (500 MHz; $CDCl_3$) 1.51 (H, *dd*, $J_{2'\text{-}3'a}$ = 5 Hz, $J_{3'\text{-}3'b}$ = 12.5 Hz, H-3′a), 2.17 (H, *dd*, $J_{2'\text{-}3'b}$ = 8 Hz, $J_{3'a\text{-}3'b}$ = 12.5 Hz, H-3′b), 4.22 (H, *dd*, $J_{2'\text{-}3'a}$ = 5 Hz, $J_{2'\text{-}3'b}$ = 8 Hz, H-2′); $\delta_{^2H}$ (61.4 MHz; $MeNO_2$) 2.4 (^{2}H-4′a) 2.5 (^{2}H-4′b); δ_C (125 MHz; $CDCl_3$) 40.09 (Me), 34.91 (C-3′), 61.93 (C-4′), 68.63 (C-2′), 175.01 (C-5′); GC-MS (rel. int.) *m/z* 178 (28), 177 (9), 150 (4), 119 (15) 100 (100) and 78 (6): enrichment = 91.8%.

[4′,4′,5′,5′-$^2H_4$]*Nicotine.* To a gently stirred soln of [4′,4′-$^2H_2$]cotinine (2 g, 11 mmol) in dry Et_2O (50 ml) in ice, was added dropwise $LiAlD_4$ (0.5 g, 13 mmol, in 50 ml dry Et_2O). After 15 min, the soln was filtered, the white ppt. washed with dry Et_2O and the pooled organic phase reduced *in vacuo* to yield a pale brown oil, confirmed [22–24] to be [4′,4′,5′,5′-$^2H_4$]nicotine; δ_H (500 MHz; $CDCl_3$) 1.63 (H, *dd*, $J_{2'\text{-}3'a}$ = 9 Hz, $J_{3'a\text{-}3'b}$ = 12.8 Hz, H-3′a), 2.06 (Me), 2.07 (H, *dd*, $J_{2'\text{-}3'b}$ = 8 Hz, H-3′b), 2.98 (H, *dd*, $J_{2'\text{-}3'b}$ = 8 Hz H-2′); $\delta_{^2H}$ (61.4 MHz; $MeNO_2$) 1.89 (^{2}H-4′a) 1.98 (^{2}H-4′b), 2.46 ^{2}H-5′a) 3.30 (^{2}H-5′b) [23]; GC-MS (rel. int.): *m/z* 166 (14), 165 (12), 135 (20), 119 (6), 88 (100) and 78 (3): enrichment = 83.1%.

Alkaloid separation and identification. Alkaloids were extracted by grinding tissue in 0.2 M H_2SO_4 with an Ultraturrax®. After *ca* 1 hr, debris was removed by filtration *in vacuo*, the soln basified (35% NH_3 soln) and applied to an Extrelute® column (20 g), previously wetted with 1 ml 35% NH_3 soln. After 15 min, the column was eluted with 75 ml of CH_2Cl_2 followed by 25 ml of CH_2Cl_2–MeOH (10:1) yielding a crude total alkaloid fr.

GC-MS. Alkaloids were sepd by GC on a 25 m Ultra-1 capillary column (0.32 mm i.d., 0.17 μm film thickness) and peaks identified by mass, R_t and fragmentation pattern [22].

TLC. The crude alkaloid extract was sepd on silica gel 60 plates (Merck) using toluene–Me_2CO–MeOH–25% NH_3 soln (8:9:2:1). R_f Values: nicotine 0.88, anatabine 0.65, cotinine 0.55, nornicotine 0.39. The frs were recovered by elution of the silica gel with

MeOH–35% NH_3 soln (9:1) and their identity and purity confirmed by GC-MS. The fr. at R_f 0.39 was shown to be [4′,4′,5′,5′-$^2H_4$]nornicotine; δ_H (500 MHz; $CDCl_3$) 1.87 (H, *dd*, $J_{2'\text{-}3'a} = 8.5$ Hz, $J_{3'a\text{-}3'b} = 11.5$ Hz, H-3′a), 2.29 (H, *dd*, $K_{2'\text{-}3'b} = 6.5$ Hz, $J_{3'a\text{-}3'b} = 11.5$ Hz, H-3′b), 2.89 (H, *br s*, N-H), 4.35 (H, *dd*, $K_{2'\text{-}3'a} = 8.5$ Hz, $J_{2'\text{-}3'b} = 6.5$ Hz, H-2′); $\delta_{^2H}$ (61.4 MHz; $MeNO_2$) 1.84 (^{2}H-4′a), 1.92 (^{2}H-4′b), 3.16 (^{2}H-5′a), 3.24 (^{2}H-5′b) [23]; GC-MS (rel. int.) *m/z* 152 (24), 151 (37), 122 (29), 121 (100), 78 (10), 74 (80).

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