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Nicotine demethylation in *Nicotiana* cell suspension cultures: *N*′-formylnornicotine is not involved

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Abstract

Nicotine or nornicotine enriched with stable isotopes in either the *N*′-methyl group or the pyrrolidine-N were fed to *Nicotiana plumbaginifolia* suspension cell cultures that do not form endogenous nicotine. The metabolism of these compounds was investigated by analysing the incorporation of isotope into other alkaloids using gas chromatography–mass spectroscopy (GC–MS). Nicotine metabolism primarily resulted in the accumulation of nornicotine, the *N*′-demethylation product. In addition, six minor metabolites appeared during the course of nicotine metabolism, four of which were identified as cotinine, myosmine, *N*′-formylnornicotine and *N*′-carboethoxynornicotine. While cotinine was formed from [^{13}C,^{2}H$_3$-methyl]nicotine without dilution of label, *N*′-formylnornicotine was labelled at only about 6% of the level of nicotine and *N*′-carboethoxynornicotine was unlabelled. Feeding with [1′-^{15}N]nornicotine resulted in incorporation without dilution of label into both *N*′-formylnornicotine and *N*′-carboethoxynornicotine. This pattern strongly indicates that, while nornicotine and cotinine are derived directly from nicotine, *N*′-formylnornicotine and *N*′-carboethoxynornicotine are metabolites of nornicotine. Thus, it is directly demonstrated that *N*′-formylnornicotine is not an intermediate in nicotine demethylation.
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Keywords: *Nicotiana plumbaginifolia*; Solanaceae; Nicotine; Nornicotine; *N*′-Formylnornicotine; Demethylation

1. Introduction

Nicotine (Fig. 1) is a naturally occurring alkaloid found primarily in members of the Solanaceae (Leete, 1983). The bioconversion of nicotine to nornicotine has been investigated over the last several decades mainly in whole plants but also in plant cell cultures. While it has been acknowledged for some time that nornicotine is derived from the demethylation of nicotine (Dawson, 1945), the exact mechanism of this process remains unclear. Early studies on whole plants of *N. tabacum* and *N. rustica* (Leete and Bell, 1959; Tso and Jeffery, 1961) as well as cell suspension cultures of *N. tabacum* and *N. glauca* (Barz et al., 1978; Hobbs and

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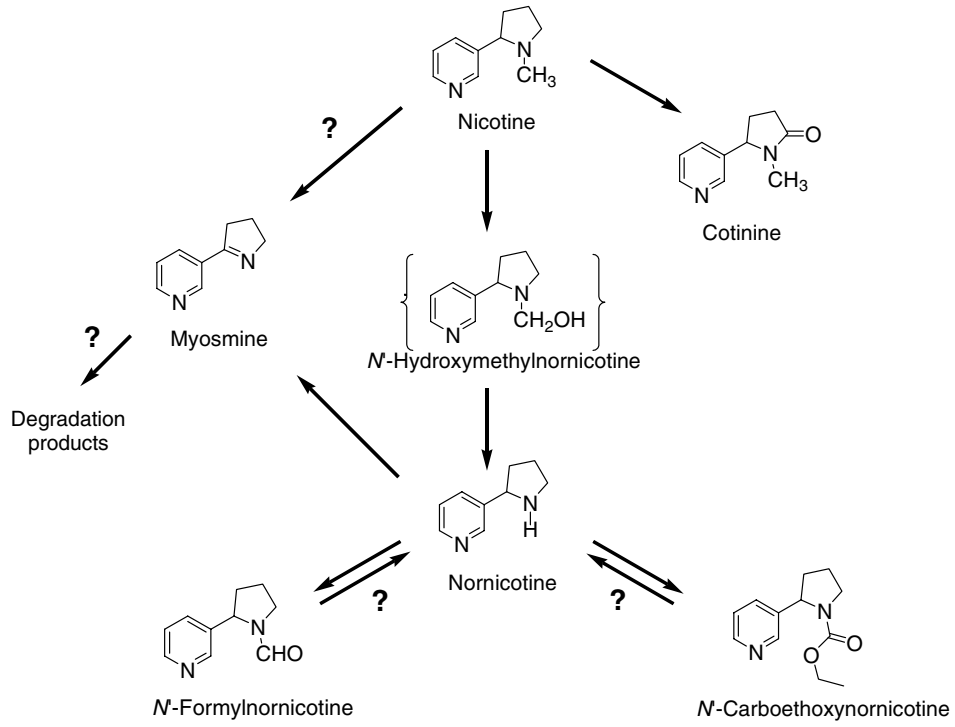

Fig. 1. Structures of the alkaloids identified in *N. plumbaginifolia* culture extracts fed nicotine or nornicotine and their proposed metabolic relationship.

Yeoman, 1991) showed that label from [^{14}C-methyl]nicotine was incorporated into a range of soluble and insoluble metabolites. This led to the hypothesis that transmethylation occurs, a proposal further supported by the apparent conversion of nicotine to nornicotine only in the presence of methyl group acceptors such as glycine or ethanolamine (Bose et al., 1956; Dyar, 1975). However, these earlier experiments have proved difficult to repeat and recent studies on the demethylation of nicotine have proposed an oxidative mechanism invoking a cytochrome P450 enzyme (Chelvarajan et al., 1993; Hao and Yeoman, 1998). Assuming this oxidative demethylation, oxidation may occur in the pyrrolidine ring at the N-1′, the C-2′ or C-5′, or on the *N*-methyl group. However, evidence has been presented that nicotine-1′-*N*-oxide is not an intermediate (Leete, 1983; Strunz and Findlay, 1985). Also, oxidation at positions C-2′ and C-5′ can be eliminated, as feeding experiments with [2′-^{14}C,^{3}H]nicotine (Leete and Chedekel, 1974) and [4′,4′,5′,5′- $^{2}H_4$]nicotine (Botte et al., 1997), respectively, indicate retention of all the label in the derived nornicotine. Hence, myosmine is formally eliminated as a possible intermediate. These results focus attention on a probable oxidation of the *N*-methyl group (Fig. 1). Experiments with [^{13}C-methyl]nicotine and [^{14}C-methyl]nicotine have shown the incorporation of the label into several amino acids and derivatives thereof (Mesnard et al., 2002), indicating that the carbon atom from the methyl group is transferred into the one-carbon metabolism pathway. This finding, which implies that the methyl moiety of nicotine passes through the tetrahydrofolate (THF) pool, is consistent with either a direct transfer of the methyl group to THF or an oxidative attack on the *N*-methyl group. Oxidation could produce *N*′-hydroxymethylnornicotine – a rather unstable compound – that would break down spontaneously to nornicotine and formaldehyde, the latter being incorporated into THF metabolism (Hanson et al., 2000; Mesnard et al., 2002). Alternatively, *N*′-hydroxymethylnornicotine could serve directly as the donor of a hydroxymethyl group to THF metabolism. A further possibility is that it undergoes additional oxidation to *N*′-formylnornicotine, a stable compound. None of these intermediates was detected by Mesnard et al. (2002), unsurprising given the relatively poor sensitivity for low-abundance metabolites of the methodology used (NMR). A more appropriate methodology for the detection of trace compounds is gas chromatography–mass spectroscopy (GC–MS). Using this approach, *N*′-formylnornicotine was identified as one of a number of unidentified metabolites of nicotine fed to cultures of *N. tabacum* (Hao and Yeoman, 1996a). However, these authors did not establish whether this product was an intermediate in nicotine demethylation or resulted from nornicotine spontaneously condensing with formaldehyde. In order to address the status of this key potential intermediate, *Nicotiana plumbaginifolia* cell suspension cultures have been fed with [^{13}C,$^{2}H_3$-methyl]nicotine,

Table 1
Metabolism of nicotine by cell suspension cultures of *Nicotiana plumbaginifolia*

Experiment no. and supplement	Time (days)	% of substrate supplied at $t = 0$ (days)														
		Nicotine		Nornicotine		Myosmine		Cotinine		*N*′-Formyl-nornicotine		*N*′-Carboethoxy-nornicotine		Total		
		M	C	M	C	M	C	M	C	M	C	M	C	M	C	M + C
1. 30 μmol unlabelled (S)-nicotine	3	34.6	11.9	30.9	3.1	nr1	1.29	1.86	0.14	nr2	0.00	0.24	0.04	67.7	16.5	84.2
	5	17.3	14.0	31.6	16.1	nr1	2.17	1.44	2.31	nr2	0.96	0.06	0.08	50.4	35.7	86.1
	6	17.2	13.5	26.0	15.3	nr1	2.31	1.43	1.52	nr2	0.74	0.14	0.11	44.7	33.5	78.3
	7	21.4	9.0	29.5	12.0	nr1	2.45	1.35	1.33	nr2	0.67	0.11	0.06	52.4	25.4	77.8
	8	12.0	8.7	24.3	17.7	nr1	1.68	1.08	1.64	nr2	0.58	0.07	0.08	37.5	30.3	67.8
	10	13.0	8.9	27.7	20.1	nr1	3.45	0.69	2.04	nr2	0.83	0.03	0.09	41.4	35.4	76.8
2. 30 μmol (*R*,*S*)-[^{13}C,$^{2}H_3$-methyl]-nicotine	3	37.2	23.6	2.3	3.8	4.38	4.14	2.07	1.32	0.36	0.85	0.82	0.58	47.1	34.2	81.3
	5	33.4	13.7	5.2	3.7	3.81	1.39	2.37	0.64	0.39	0.41	0.84	0.16	46.1	20.0	66.1
	6	34.1	8.0	9.1	3.3	2.40	1.02	2.58	0.50	0.43	0.39	0.00	0.31	48.6	13.6	62.2
	7	32.0	7.4	10.2	2.0	0.00	0.89	2.33	0.42	0.46	0.43	0.75	0.19	45.7	11.4	57.0
	8	29.9	7.2	7.8	2.7	1.55	1.06	2.01	0.60	0.39	0.44	1.15	0.20	42.7	12.2	54.9
	10	32.8	6.5	9.0	1.2	1.56	0.09	2.21	0.36	0.37	0.41	0.71	0.19	46.7	8.7	55.4
3. 30 μmol (*R*,*S*)-[1′-^{15}N]-nicotine	3	38.6	18.6	8.8	8.2	nr1	1.82	0.06	0.95	0.73	1.85	0.10	0.18	48.3	31.7	80.0
	5	22.0	1.9	13.3	6.8	nr1	1.33	0.11	0.18	1.01	0.74	0.12	0.19	36.6	11.1	47.7
	6	20.6	2.3	25.3	15.7	nr1	0.00	0.13	0.08	1.01	0.91	0.13	0.18	47.2	19.2	66.3
	7	3.1	0.2	29.7	24.1	nr1	4.10	0.14	0.09	0.85	3.33	0.21	0.20	34.1	32.0	66.1
	8	0.1	0.1	29.4	15.1	nr1	2.67	0.14	0.09	0.61	2.12	0.12	0.17	30.4	20.2	50.6
	10	0.2	0.3	24.7	9.5	nr1	3.61	0.07	0.32	0.57	2.75	0.10	0.22	25.7	16.7	42.4

Each experiment was conducted in duplicate. The error in the values given for the different metabolites is between 3% and 8%.
M, medium; C, cells; M + C, medium plus cells combined; nr1, present in small amount but not resolved from the nornicotine peak; nr2, present in small amount but not sufficiently resolved from unidentified adjacent peak.

[1′-^{15}N]nicotine and [1′-^{15}N]nornicotine and their incorporation into a range of products analysed by GC–MS. It is now directly established that *N*′-formylnornicotine is a metabolite of nornicotine.

2. Results and discussion

2.1. Metabolism of nicotine

Nicotine fed to *Nicotiana plumbaginifolia* suspension cells was effectively metabolised. Feeding was carried out using three different sources of nicotine: (*S*)-nicotine as control, (*R*,*S*)-[^{13}C,^{2}H$_3$-methyl]nicotine, and (*R*,*S*)-[1′-^{15}N-methyl]nicotine. As can be seen (Table 1), in all three feeding experiments the amount of nicotine present diminished over the time course of the experiment, and a number of metabolites could be identified (Fig. 2). These were identified by GC–MS and reference to the mass spectra of reference compounds as nornicotine (R_t, 11.58 min; *m*/*z* 148/147/119/105/70), myosmine (R_t, 11.64 min; *m*/*z* 146/145/118/105/78), cotinine (R_t, 15.02 min; *m*/*z* 176/147/119/118/98), *N*′-formylnornicotine (R_t, 15.80 min; *m*/*z* 176/159/147/119/105/70), and *N*′-carboethoxynornicotine (R_t, 15.97 min; *m*/*z* 220/191/175/147/119/105/70). In addition, two further very minor unidentified metabolites giving peaks at 16.32 min (*m*/*z* 190/175/161/147/120/119/106/70) and 18.16 min (*m*/*z* 219/191/176/175/148/147/119/106/70) were noted. Under these chromatographic conditions, nicotine had an $R_t = 10.70$ min. Of the products, nornicotine was again identified as the principle metabolite. All these alkaloids were absent at day 0 but could be detected by day 3 and persisted throughout the remainder of the 10-day experiment. As can be seen from these three examples, the kinetics of nicotine metabolism is rather variable but the metabolic profile is reproduced in each case. All these alkaloids were found in both the extra-cellular and intra-cellular compartments. However, as previously observed (Manceau et al., 1989; Mesnard et al., 2001), the total present did not correspond to the total nicotine metabolised (Table 1), indicating that nornicotine is further metabolised. In order to establish the relationship between nicotine and these metabolites, the incorporation of label from enriched nicotine into the different products was determined by GC–MS.

The incorporation of label from (*R*,*S*)-[^{13}C,^{2}H$_3$-methyl]nicotine into all the identified metabolites of nicotine was analysed. Recovered nicotine was labelled at 98.3 ± 0.7%, showing no dilution and confirming the lack of synthesis by these cultures. Cotinine was labelled at 97.8 ± 0.6% in the ^{13}C^{2}H$_3$-methyl group, consistent with a direct production from the fed nicotine. The position of the label could be deduced from the mass spectrum of this labelled compound (Fig. 3), which showed a molecular ion at *m*/*z* 180, and other major fragments at 123 and 102 (Fig. 3(a)). This fragmentation pattern corresponded accurately to the MS of an unlabelled cotinine standard, which showed ions at 176 (molecular ion), 119 and 98 (Fig. 3b) and with the established fragmentation patterns for unlabelled cotinine (Duffield et al., 1965) and for [4′,4′-^{2}H$_2$]cotinine (Botte et al., 1997). It has been observed previously that [4′,4′-^{2}H$_2$]cotinine is an oxidative metabolite of [4′,4′,5′,5′-^{2}H$_4$]nicotine in root cultures of *N. alata* but cannot be an intermediate in the demethylation reaction since [4′,4′,5′,5′-^{2}H$_4$]nicotine gave rise to [4′,4′,5′,5′-^{2}H$_4$]nornicotine (Botte et al., 1997). In man, cotinine is a major product of the hepatic oxidative metabolism of nicotine (Gorrod and Schepers, 1999). By analogy with the mammalian oxidative pathway, the formation of cotinine could occur via an iminium ion intermediate (Nguyen et al., 1979; Peterson and Castagnoli, 1988), consistent with the suggestion

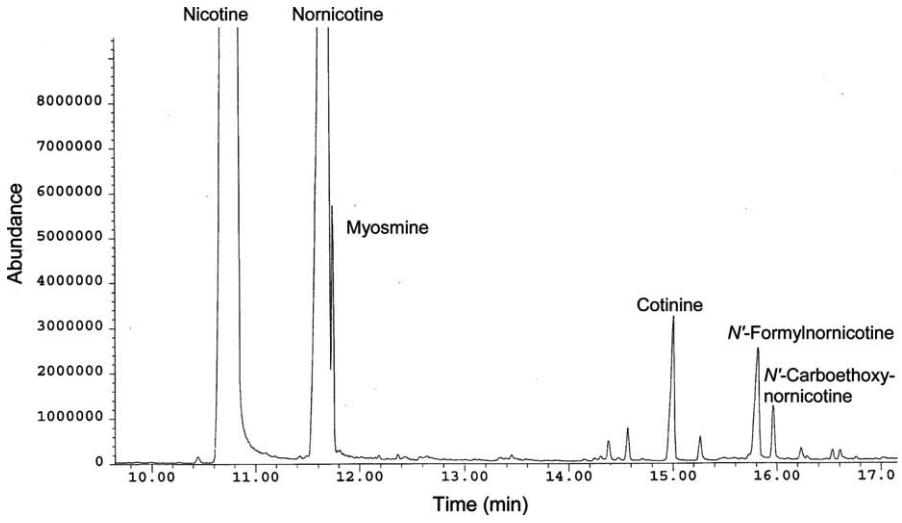

Fig. 2. GC profile of alkaloids extracted from cell cultures of *N. plumbaginifolia* after 5 days feeding with [^{13}C^{2}H$_3$-methyl]nicotine.

that oxidation of nicotine in plants involves a cytochrome P450 system.

As expected, nornicotine and myosmine were unlabelled, coherent with their loss of the methyl group. Myosmine, a minor tobacco alkaloid found in *Nicotiana* species, is thought to arise from nornicotine degradation (Leete and Chedekel, 1974) and is probably further degraded to nicotinic acid (Kisaki and Tamaki, 1966). To what extent the myosmine pathway represents an important route for the degradation of nornicotine remains to be elucidated. In cultures of *N. plumbaginifolia*, this product does not accumulate to any appreciable extent but such a role is compatible with the consistently observed loss of alkaloid from the system (present data and (Mesnard et al., 2001)).

N′-Formylnornicotine, in contrast, showed some retention of label. Critically, however, this compound was only labelled in the M + 2 ion at 6.2 ± 1.2%, an incorporation much below that of the original nicotine or the cotinine derived directly therefrom. If *N*′-formylnornicotine were a degradation product of nicotine in which the *N*-methyl bond was unbroken, then the formyl moiety should retain ^{13}C at a similar level to cotinine and, depending on the extent of exchange with the medium, one ^{2}H atom. An insignificant enrichment at the M + 1 was found, which indicates that H-exchange

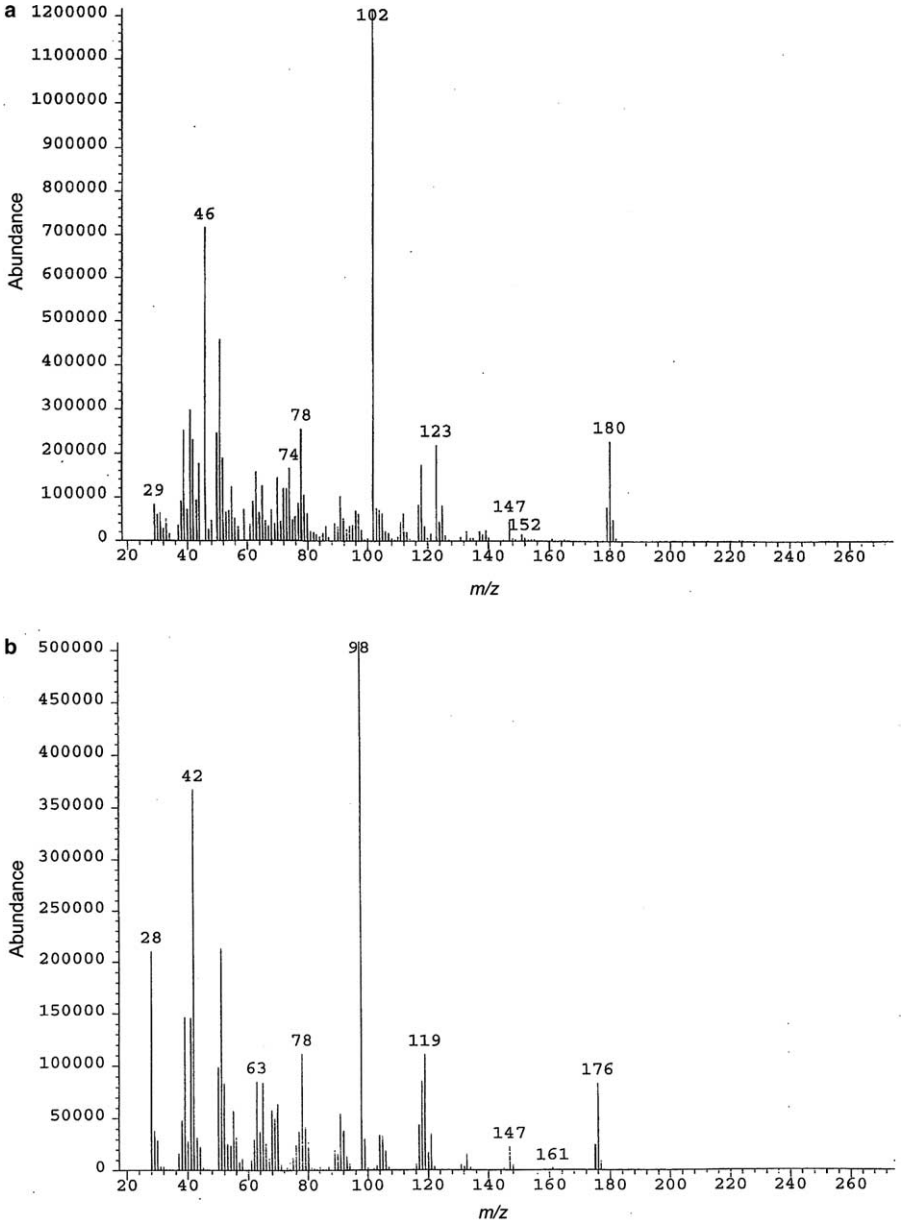

Fig. 3. Mass spectrum of labelled cotinine (a) and unlabelled cotinine (b) from cultures fed [$^{13}C^{2}H_3$-methyl]nicotine and unlabelled nicotine respectively.

Table 2
Metabolism of nornicotine by cell suspension cultures of *Nicotiana plumbaginifolia*

Experiment no. and supplement	Time (days)	% of substrate supplied at $t = 0$ (days)												
		Nornicotine		Myosmine		Cotinine		*N*′-Formyl-nornicotine		*N*′-Carboethoxy-nornicotine		Total		
		M	C	M	C	M	C	M	C	M	C	M	C	M + C
4. 32 μmol unlabelled (*R*,*S*)-nornicotine	3	55.7	46.8	nr1	nr1	0	0	0.09	0.47	nr2	0.06	55.8	47.4	103.1
	5	45.0	52.1	nr1	nr1	0	0	0.10	0.53	0.04	0.05	45.1	52.7	97.7
	6	51.3	51.9	nr1	nr1	0	0	0.11	0.57	0.02	0.07	51.4	52.6	104.0
	7	51.7	39.1	nr1	nr1	0	0	0.00	0.57	nr2	0.05	51.7	39.7	91.4
	8	53.0	29.4	nr1	nr1	0	0	0.00	0.82	nr2	0.05	53.0	30.3	83.3
	10	61.9	28.7	nr1	nr1	0	0	0.06	0.27	nr2	3.70	61.9	32.6	94.6
5. 32 μmol (*R*,*S*)-[1′-^{15}N]-nornicotine	3	75.2	0.1	nr1	2.16	0	0	5.01	0.09	4.24	0.13	84.5	2.6	87.1
	5	72.1	0.1	nr1	1.89	0	0	2.62	0.08	3.59	0.06	78.4	2.2	80.5
	6	75.8	0.1	nr1	1.20	0	0	1.34	0.17	5.67	0.12	82.8	1.6	84.4
	7	73.9	0.4	nr1	2.65	0	0	0.96	0.16	6.22	0.20	81.1	3.5	84.6
	8	60.3	4.2	nr1	5.10	0	0	2.18	0.34	7.63	0.42	70.1	10.1	80.2
	10	39.3	8.1	nr1	9.32	0	0	0.53	0.75	7.39	1.02	47.2	19.4	66.6

Each experiment was conducted in duplicate. The error in the values given for the different metabolites is between 3% and 8%.
M, medium; C, cells; M + C, medium plus cells combined; nr1, present in small amount but not resolved from the nornicotine peak; nr2, present in small amount but not sufficiently resolved from unidentified adjacent peak.

was minimal. It is evident that the level of labelling determined is incompatible with *N*′-formylnornicotine being intermediate to the demethylation of nicotine to nornicotine, eliminating the possibility that this is an intermediate prior to the scission of the N–C bond (Fig. 1). Although it has previously been speculated that *N*′-formylnornicotine arises by the condensation of nornicotine and formaldehyde (Hao and Yeoman, 1996b; Hobbs and Yeoman, 1991), these authors provided no direct evidence to support this hypothesis. The present evidence now formally and directly eliminates *N*′-formylnornicotine as an intermediate in nicotine demethylation.

The conclusion that *N*′-formylnornicotine arises by the condensation of nornicotine and formaldehyde is supported by two further pieces of evidence. Firstly, the low but significant labelling of *N*′-formylnornicotine is consistent with the low labelling previously observed in a range of compounds deriving a C1 unit from the THF pool (Mesnard et al., 2002). The conversion of the labelled methyl moiety of nicotine to labelled formaldehyde, which is diluted in the C1 pool (Hanson et al., 2000), is consistent with observations that substantial ^{14}C is recovered in carbon dioxide from cell cultures fed with [^{14}C-methyl]nicotine (Barz et al., 1978; Mesnard et al., 2002). Thus, it is evident that *N*′-formylnornicotine is similarly derived by condensation of nornicotine with formaldehyde. It should be noted that the *N*′-formylnornicotine and the *N*′-carboethoxynornicotine only occur at about 0.5 to 2% of the level of nornicotine (Table 1), levels perfectly consistent with this origin. Secondly, the minor metabolite *N*′-carboethoxynornicotine – previously found in flue-cured tobacco (Matsushita et al., 1979) – would logically be derived from nornicotine by appropriate condensation with the secondary amine of the pyrrolidine ring. This compound shows no enrichment, as expected in view of its proposed biosynthetic origin.

To confirm that all the identified compounds were derived from the fed nicotine, incorporation from (*R*,*S*)-[1′-^{15}N]nicotine ($11.3 \pm 0.9\%$ atom enrichment) was also examined (Table 1). Recovered nicotine was labelled at $11.8 \pm 1.7\%$ and all the derived metabolites showed values between 11% and $13 \pm 1.5\%$.

2.2. Metabolism of nornicotine

Further evidence in support of this conclusion was sought by examining the metabolism of nornicotine by cell suspension cultures of *N. plumbaginifolia* in feeding experiments in which (*R*,*S*)-nornicotine or (*R*,*S*)-[1′-^{15}N]nornicotine were fed to cultures (Table 2). As can be seen, and as implied by previous observations (Mesnard et al., 2001), nornicotine can also be metabolised, myosmine, *N*′-formylnornicotine, and *N*′-carboethoxynornicotine all being identified in the cultures. As is to be expected, the *N*-methyl compounds cotinine (Table 2) and nicotine (data not shown) were notably absent. Furthermore, no norcotinine was found by GC–MS, indicating the importance of the presence of the *N*-methyl group for this α-oxidation.

Label from (*R*,*S*)-[1′-^{15}N]nornicotine ($11.8 \pm 0.5\%$ atom enrichment) was incorporated into all the metabolites identified. Recovered nornicotine was labelled at $11.4 \pm 1.7\%$, *N*′-formylnornicotine at $10.0 \pm 0.3\%$, and *N*′-carboethoxynornicotine at $12.9 \pm 0.3\%$. This confirmed that these products were derived from the fed nornicotine and not from some other source. Incorporation of ^{15}N into myosmine could be detected but not quantified due to the overlap of the large nornicotine peak with the small myosmine peak in the GC–MS chromatogram (Fig. 2).

Hence, the metabolism of nornicotine confirms that the *N*′-formylnornicotine and the *N*′-carboethoxynornicotine are derived from nornicotine and not by direct metabolism of nicotine. It cannot, however, be established whether myosmine is derived exclusively from nornicotine or that a direct route from nicotine also exists (Fig. 1).

3. Conclusion

[^{13}C,$^{2}H_3$-methyl]Nicotine fed to *Nicotiana plumbaginifolia* cell cultures gives rise to cotinine, which retains fully the ^{13}C and ^{2}H enrichment of the substrate, and to *N*′-formylnornicotine with substantially diminished but significant enrichment. This is convincing evidence that the latter compound is not an intermediate in the process of nicotine demethylation. Rather, it would appear to be one of a range of *N*-acyl derivatives of nornicotine known to occur in *Nicotiana* plants (Leete, 1983), a view supported by the incorporation of ^{15}N-label from (*R*,*S*)-[1′-^{15}N]nornicotine.

It is increasingly apparent that nornicotine is a key compound in the defensive mechanism of *Nicotiana* plants, nicotine acting to supply nornicotine to be converted into compounds some of which are highly toxic to invasive herbivores (Laue et al., 2000). While some nornicotine derivatives, such as the *N*′-formylnornicotine and *N*′-carboethoxynornicotine identified in the present study, probably arise through spontaneous condensation of the reactive secondary amine of nornicotine, the indication is that the formation of defensive *N*-acylnornicotine compounds is regulated in response to herbivory (Laue et al., 2000). The regulation of this process and the underlying enzymology merits further attention.

The present study, while advancing our overall understanding of the important metabolism that leads to nicotine turnover in plants, also narrows the possibilities for the means of nicotine demethylation. It is

increasingly evident that the most probable mechanism is an oxidative elimination of the *N*′-methyl group of nicotine, a putative *N*′-hydroxymethylnornicotine spontaneously decomposing into nornicotine and formaldehyde (Fig. 1). That this putative intermediate does not undergo further oxidation to produce *N*′-formylnornicotine is evidenced by the level of label observed in this latter compound. Further support for this proposed route is obtained from determining the kinetic isotope effect in ^{15}N on the demethylation reaction, which indicates that C–N bond fission is spontaneous and rapid (Molinié, 2005 and unpublished data).

4. Experimental

4.1. Chemicals

(*S*)-Nicotine (purity > 98%) was obtained from Sigma Chemical Co. (www.sigmaaldrich.com) and (*R*,*S*)-nornicotine (purity > 96%) from Lancaster Synthesis (www.lancastersynthesis.com).

[^{13}C,$^{2}H_3$-methyl]Nicotine (98.3 ± 0.7% atom enrichment) was synthesised as described previously (Girard, 2000; Mesnard et al., 2001; Mesnard et al., 2002). [1′-^{15}N]Nicotine (11.3 ± 0.9% atom enrichment) and [1′-^{15}N]nornicotine (11.8 ± 0.5% atom enrichment) were synthesised as described previously (Vo-Thanh et al., 2001).

4.2. Plant material and culture conditions

Cell suspension cultures of *Nicotiana plumbaginifolia* were used in the experiments. These cells do not synthesise nicotine but are able to demethylate exogenous nicotine effectively (Manceau et al., 1989; Mesnard et al., 2001). The *N. plumbaginifolia* cells were grown in 'FMD' medium (Mesnard et al., 2002), and were maintained in an incubator at 25 °C, on a gyratory shaker at 100 rpm, under constant illumination. The cells were subcultured into new medium every fortnight.

4.3. Feeding experiments

The feeding experiments were carried out in six-well sterile plates, each well containing 2 g fresh weight of cells in the exponential growth phase, 30–35 μmol labelled or unlabelled substrate, and 5 ml of sterile 'FMS' medium (Mesnard et al., 2002). A control feed was done under identical conditions, without any addition of alkaloid, keeping the osmolarity of the solution equivalent with sodium chloride. The cultures were incubated at 25 °C, on a gyratory shaker at 100 rpm, under constant illumination. After 3–10 days, the cells were harvested, separated from the medium, and washed with 5 ml of deionised water. The cells and the pooled culture media plus washings were conserved in a freezer at −20 °C until extraction.

4.4. Extraction procedures

To extract the tobacco alkaloids, the cells were homogenised with an Ultra-Turrax® tissue disintegrator (IKA Labortechnik, Janke & Kunkel, D-71219 Staufen, Germany) and extracted with 25 ml of chloroform:ammonia soln. (30%) (24:1 v/v) under reflux for 2 h. The extract was filtered and evaporated to dryness at 30 °C under reduced pressure. Analysis was carried out on this extract. It was confirmed in control experiments that this protocol did not lead to the formation of any of the metabolites identified by GC–MS in the cell extracts.

The culture media were basified with 2 ml of ammonia soln. (30%) and the alkaloids were extracted three times with 10 ml of chloroform. The extract was evaporated to dryness at 30 °C under reduced pressure.

The crude extracts were taken into 200 μl of chloroform and the solution dried with anhydrous sodium sulphate.

4.5. GC–MS experimental conditions

GC–MS was performed using a Hewlett Packard (www.hp.com) 5890 gas chromatograph directly connected to a Hewlett Packard 5972 Mass Selective Detector quadrupole mass spectrometer. The column used for GC separation was a DB5 capillary column (J&W Inc., 30 m × 0.32 mm i.d. × 0.25 μm film thickness). The thermal elution conditions were: 100 °C for 2 min; 5 °C/min to 240 °C. The carrier gas was He (1 ml/min constant flow) and the injection volume was 1 μl. Mass spectra were collected using electron impact ionisation at 70 eV. The injection and detector temperatures were 250 °C.

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