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MECHANISM OF NICOTINE *N*-DEMETHYLATION IN TOBACCO CELL SUSPENSION CULTURES

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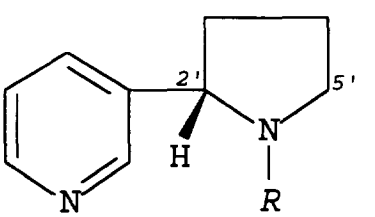
Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco cell culture; *N*-demethylation; nicotine; nornicotine.

Abstract—Radioactive feeding experiments, in which DL-[pyrrolidine-2′-^{14}C]-nicotine was added to 10-day-old cultures, confirmed that the kinetic pattern of this *N*-demethylation was similar to that of non-radioactive nicotine, and that nornicotine, the major product, was produced intracellularly with a maximum percentage conversion of about 70%. The appearance of nornicotine paralleled the disappearance of the added nicotine, although small amounts of radioactive metabolites other than nornicotine were also observed. One of these metabolites was tentatively identified by GC-MS as *N*-formyl-3′-nornicotine which is probably a side-product rather than an intermediate in nicotine *N*-demethylation. Nornicotine produced from added (−)-nicotine by the cultures was determined using both polarimetry and chiral GC, and shown to be exclusively one enantiomer. This provided strong evidence that the mechanism of this reaction in cultured cells was unlikely to involve opening of the pyrrolidine ring and, therefore, differed from that previously hypothesized for the tobacco plant. The possible mechanism for this reaction is also discussed.

INTRODUCTION

It is well known that the bioconversion of nicotine to nornicotine in tobacco leaves during the course of air curing and fermentation is a natural phenomenon of commercial significance [1]. The change of nicotine and nornicotine content in tobacco can seriously affect the quality of tobacco products, since nornicotine contributes a harsh and bitter flavour to cigarettes [2].

This bioconversion of nicotine to nornicotine has been investigated over the last decade mainly in the plant but also in cell cultures. The present state of knowledge, including assumptions, of this reaction was comprehensively reviewed by Wallex and Nowacki [1] and Poulton [3]. An important hypothetical scheme of this reaction in the tobacco plant was published by Leete and Chedekel in 1974 [4] based on the observation [5] that racemized nornicotine was produced from (−)-nicotine (**1**) by excised *Nicotiana* leaves. According to this hypothesis, (−)-nicotine is first converted to (−)-nicotine-1′-oxide, which would provide a hydroxyamine via a Cope elimination resulting in the opening of the pyrrolidine ring (hence disappearance of the optical isomerism). Further elimination of water from this hydroxyamine yields the Schiff base, which upon hydrolysis produces formaldehyde or another C1 metabolite and a primary amine. Final cyclization of this primary amine gives rise to (+)-and (−)-nornicotine (**2**). However, there has been no substantial evidence reported over the last 20 years to support this hypothesis. Nor has any real attempt been made to investigate a reaction mechanism involving the removal of a methyl group from the pyrrolidine ring moiety of nicotine (**1**).



R	
1	Me
2	H
3	CHO

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It is now well established that plant cell cultures are capable of converting a variety of added chemicals with high efficiency [6]. Using tobacco cell cultures, contributions have been made towards understanding the nature of nicotine *N*-demethylation. Barz *et al.* [7] in studies on the degradation of nicotine in *Nicotiana* cultures reported that the degree of *N*-demethylation of added nicotine depended on the auxin level employed. Indeed it has been shown that cell cultures of *Nicotiana tabacum* L. cv Wisconsin-38 convert added nicotine to nornicotine as the predominant product [8]. Thus, this culture line was selected for the investigation of nicotine-*N*-demethylation. In this paper, which is a continuation of the work of Hobbs and Yeoman [8], the reaction mechanism has been studied using radioactive tracers and stereochemical techniques.

RESULTS AND DISCUSSION

Distribution of radioactivity in the cultures

A 1 μCi sample of ^{14}C-nicotine was added to 10 ml aliquots of a randomized mixture of 10-day-old cell cultures and incubated under standard culture condition for 24 hr. Extraction of the alkaloids from these cultures resulted in three fractions. The results presented in Table 1 show that 92% of the added radioactivity can be accounted for with about 75% in the chloroform phase, 15% in the aqueous phase and 2.7% in the cell debris. The 8% of added radioactivity not accounted for was probably absorbed onto the glassware during the lengthy extraction process. The 75% recovery of added ^{14}C-nicotine is similar to the values reported for the recovery of added non-radioactive nicotine (81 $\pm$4% [9]). Accordingly, the experimental data were corrected before kinetic analysis.

[2′-^{14}C]-*Nicotine feeding experiments*

Aliquots 10 ml of a randomized mixture of 10-day-old cell cultures were transferred aseptically to each of 50 ml conical flasks, to which 0.5 μCi ^{14}C-nicotine was added, and incubated under standard culture conditions. Three flasks were sampled at 1, 6, 12, 24, 48 and 72 hr and the alkaloids extracted. Subsequently, the extracted alkaloids were separated by TLC. All labelled compounds on the TLC plate were first visualized by autoradiography and then scraped off and counted.

Figure 1 is an autoradiograph of a TLC plate illustrating the bioconversion of nicotine to nornicotine. It can be seen that radioactive nornicotine appears 1 hr after addition of nicotine in both cells and medium and has increased, by 6 hr, particularly in the cells. The decrease in radioactivity of nicotine drops sharply in the medium at 6 hr with a corresponding increase in the cells. After 6 hr nicotine content in both cells and medium gradually tends to be constant. The kinetics of bioconversion shown in Fig. 2 confirms the results from the autoradiograph, that the bioconversion ^{14}C-nicotine had begun within 1 hr of addition and increased dramatically over the first 6 hr. Thereafter, there was a steady increase until 48 hr when 70.3% of the total recoverable nicotine had been converted.

It can be seen from Figs 3(a) and (b) that labelled nornicotine appears in the cells within 1 hr when more

Table 1. Distribution of radioactivity in the cultures 24 hr after the addition of ^{14}C-nicotine

Fractions	Radioactivity (dpm $\times 10^{-5}$)	Recovery (%)*
Chloroform	16.6 $\pm$ 0.2	74.8
Aqueous†	3.2 $\pm$ 0.2	14.5
Cell debris	0.6 $\pm$ 0.1	2.7
Total recovered	20.4 $\pm$ 0.9	92.0

*Recovery (%) is the percentage of radioactivity extracted in each fraction as a proportion of that added.

†Aqueous fraction is the aqueous phase of the cultures after extraction with $CHCl_3$.

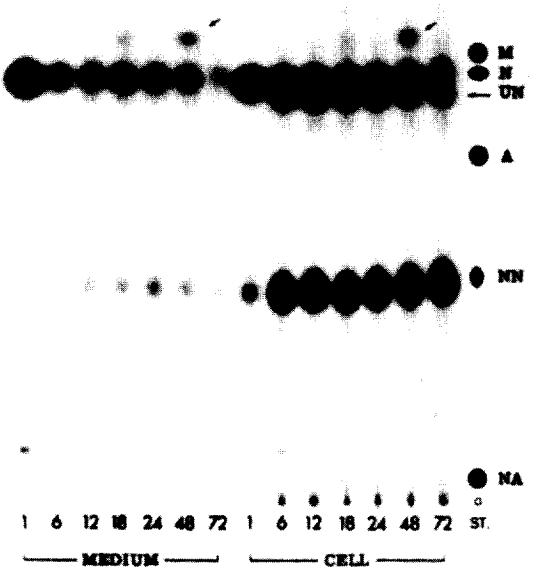

Fig. 1. Autoradiograph of a TLC plate showing a 72 hr time course of the bioconversion of ^{14}C-nicotine to ^{14}C-nornicotine. Numbers beneath the loading points indicate the sampling times (hr) after the addition of ^{14}C-nicotine to a 50 ml flask containing 10 ml of a 10-day-old cell culture. N, nicotinc; NN, nornicotine; UN, unknown compounds; NA, nicotinic acid; M, myosmine, A, anatabine; and St., a mixed sample of non-radioactive authentic standards which were drawn according to their spots visualized by the method described after autoradiography.

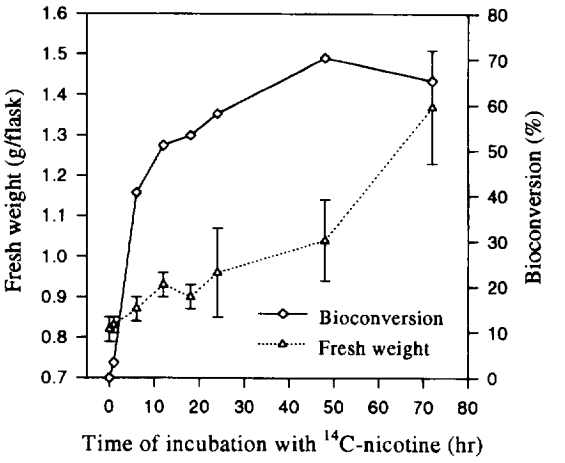

Fig. 2. Change in fresh weight and kinetics of the bioconversion of ^{14}C-nicotine added 10 days after subculture.

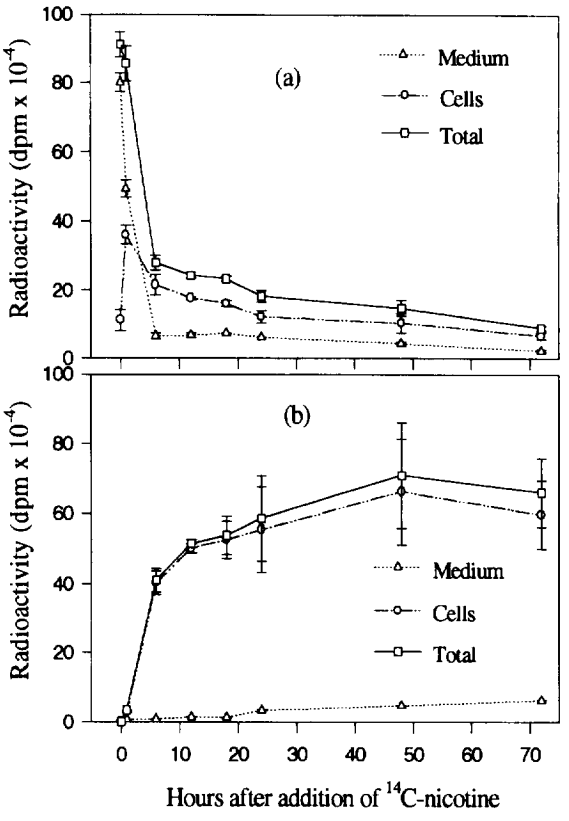

Fig. 3. Change in the radioactivity in (a) nicotine and (b) nornicotine in cells and medium after the addition of ^{14}C-nicotine (0.5 μCi, 9.5×10^{-3} μmol) 10 days after subculture.

than 40% of the added ^{14}C-nicotine has been taken up. These results are similar to those reported by Barz and his co-workers [7]. During the first 6 hr more than 80% of the added radioactivity has disappeared from the medium (Fig. 3a). This loss can be partially accounted for by uptake into cells, since radioactivity in the cell fraction increased significantly within the first hour of incubation before decreasing. After 1 hr, the amount of ^{14}C-nornicotine increased sharply, paralleling the decrease in ^{14}C-nicotine until an equilibrium was reached at around 6 hr. However, the balance between the disappearance of nicotine and appearance of nornicotine was not the same as that reported by Hobbs and Yeoman [8] with non-radioactive nicotine, presumably because small amounts of several compounds other than nornicotine were labelled. It was also observed that most of the ^{14}C-nornicotine produced appeared in the cell fraction rather than in the medium at all sampling times. This is consistent with the observations of Barz and his co-workers [7] with ^{14}C-nicotine, but not with the observation of Hobbs and Yeoman [8] with non-radioactive nicotine who showed that 93% of the alkaloids were released into the medium. This difference could be related to the fact that the added ^{14}C-nicotine (9.5×10^{-3} μmol) was diluted out considerably by endogenous nicotine, since the concentration of added ^{14}C-nicotine was only approximately 1/160th that of endogenous nicotine. This 'diluting effect' could also explain why only 70% of added ^{14}C-nicotine was converted into ^{14}C-nornicotine by the end of the incubation. Nevertheless, these results do support the suggestion of Hobbs and Yeoman [8] that the bioconversion of nicotine to nornicotine is intracellular.

Identification of unknown metabolites

It can be seen from Fig. 1 that several labelled compounds other than nicotine and nornicotine appeared in the cell fraction 6 hr after the addition of ^{14}C-nicotine and persisted until the end of the experiment. Some of these compounds with a lower R_f than the unknown compound (UN) are due to impurities in the ^{14}C-nicotine (figure not shown). In order to establish whether any of these labelled compounds are intermediate(s) of nicotine bioconversion to nornicotine, 10 mg of (±)-nicotine (Sigma) was added to each of five 250 ml conical flasks containing 50 ml of a 10-day-old cultures, which were then incubated for 72 hr. After removing cells from medium, the alkaloids in the cell fraction were extracted and separated by preparative TLC. The spots with a higher R_f than the UN (see Fig. 1) were located on a TLC plate by co-elution with a 48 hr radioactive sample from the radioactive feeding experiment. The area coinciding with those labelled compounds was subsequently scraped off the TLC plate, extracted with methanol and analysed by GC-MS.

Four peaks from the methanol extracts were recorded by GC-MS. On the basis of authentic GC-MS data, one of the peaks was identified as nicotine, because it presents the same key diagnostic fragments (m/z) and a very similar relative intensity as nicotine (e.g. 162 (61), 161 (60), 133 (66), 119 (33), 84 (100), 78 (32)), indeed it also shows a GC retention time (4.36 min) corresponding to the nicotine standard (4.43 min). However, the identities of the other two peaks remain unknown. It can be deduced from the possible chemical structure that the

fourth peak may be a molecule with a formula of $C_{10}H_{12}N_2O$. In its mass spectral fragmentation, 176 $[M]^+$ (100) represents the parent ion; 158 (18) represents $[M - H_2O]^+$ and 147 (85.1) represents $[M - CHO]^+$. There are also two fragments, 98 (50) and 78 (28), assigning [formylpyrrolidine moiety]$^+$ and [pyridine moiety]$^+$, respectively. In addition, the mass spectral fragmentation pattern of this peak is consistent with that of *N*-formyl-3′-nornicotine (**3**) as described by Braumann *et al.* (pers. commun.) with the same GC retention time of 13.1 min. Accordingly, the compound of this peak was tentatively identified as *N*-formylnornicotine (**3**) on the basis of GC-MS data.

N-Formylnornicotine has been isolated from Burley tobacco [10] and has also been found in tobacco cell cultures at the end of a 30-day incubation with nicotine [8]. It has been established that oxidative elimination of the *N*-methyl group of a substrate initiated by hydroxylation to form a hydroxymethyl derivative may be spontaneously decomposed into product and formaldehyde [11]. The resulting hydroxymethyl derivative may also undergo further oxidation leading to *N*-formylnornicotine [12]. Alternatively, the resulting formaldehyde may react with the nornicotine formed to produce *N*-formylnornicotine. Indeed, this could occur spontaneously, since the reaction between a –NH group and formaldehyde occurs readily in organic synthesis. From this point of view, *N*-formylnornicotine would appear to be a side-product rather than an intermediate in nicotine *N*-demethylation. However, further evidence is required before a firm conclusion can be made.

Stereochemical studies

One of the important hypotheses concerning the mechanism of nicotine *N*-demethylation was made by Leete and Chedekel in 1974 [4], in which they presumed that this reaction involved the opening and subsequent closing of the pyrrolidine ring during the conversion. In order to investigate the optical properties of nornicotine derived from nicotine added to cell cultures, and to confirm if a racemization is involved in this reaction, 10 mg (–)-nicotine (Sigma) was added to 50 ml of a 10-day-old cell suspension in a 250 ml conical flask. The culture was harvested after incubation for 10 days and the alkaloids extracted. After separation on preparative TLC, nicotine and nornicotine were identified as bands under UV light, according to their respective authentic standards, and the bands scraped off the plate. The scraped silica gel was extracted with HPLC grade methanol and extracts analysed by polarimetry and chiral GC.

It can be seen from Table 2, that the specific rotation of nornicotine converted from (–)-nicotine added to the cell cultures is very close to that of pure (–)-nornicotine, suggesting that no racemization occurred during the reaction. It was also observed that the added (–)-nicotine had not been optically changed during the incubation. In order to confirm the optical property of the nornicotine converted from (–)-nicotine added to the cultures, the same samples of nornicotine were subjected to chiral GC analysis. The results obtained (shown in Fig. 4) are clearly consistent with that from polarimetry, indicating that the synthetic nornicotine is a racemate which contains 52% (+)- and 48% (–)-nornicotine, that natural nornicotine contains 25% (+)- and 75% (–)-nornicotine and that the nornicotine obtained from bioconversion by cell cultures contains (+)-nornicotine only as a minute trace.

The evidence obtained in this study shows convincingly that nornicotine produced from added nicotine is only one enantiomer, suggesting that nicotine *N*-demethylation by tobacco cell cultures does not involve ring opening which does not conform to the hypothesis of Leete and Chedekel [4]. It is currently unclear whether there are two different mechanisms, one in the plant and another in plant cell cultures, and the possibility of the existence of two mechanisms has been proposed by Stepka and Dewey [13]. Indeed, it has been suggested that the overall level of metabolic control in plant cell cultures is different from that in the plant [14], so that the physiological and biochemical characteristics of cell cultures could well differ from the intact plant. In fact, there has been no substantial evidence to support the Leete and Chedekel hypothesis. This might be why an alternative mechanism not involving the opening of the pyrrolidine ring was proposed by Leete [12], where the racemic nornicotine was converted from (–)-nicotine through a tautomeric shift, and indeed this tautomerism

Table 2. The optical rotations (°) of nicotine and nornicotine

	$[\alpha]_D^{20}$ (MeOH)		
	(–)-form	(±)-form	Sample
Nornicotine*	– 40.5 (c = 7.5)	– 26.0 (c = 7.5)	– 42.0 (c = 0.81)
Nicotine†	– 134.0 (c = 8.2)	0.0 (c = 10.0)	– 132.0 (c = 9.8)

*(–)-Nornicotine (purity >98.5%) and (±)-nornicotine (natural nornicotine, purity >91.5%) were standards provided by Rothmans International Services Ltd; the sample is the nornicotine converted from added (–)-nicotine by the cell cultures.

†(–)- and (±)-Nicotine are standards from Sigma; the sample is the nicotine left after bioconversion at the end of a 10 days incubation.

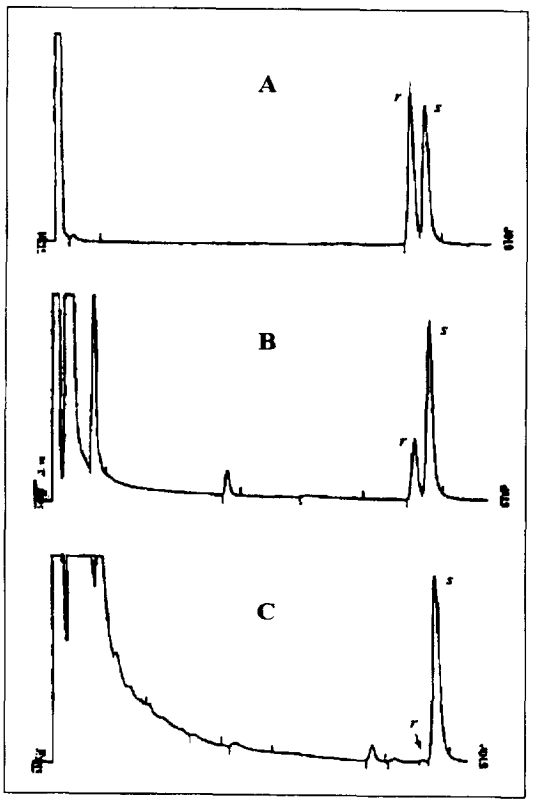

Fig. 4. Gas chromatograms of chiral analysis of nornicotine. *N*-Camphanoyl-derivatives of A, (±)-nornicotine (synthetic); B, nornicotine from Burley tobacco; and C, nornicotine derived from added (−)-nicotine by tobacco cell cultures. r, s, configurational symbols (r: *rectus* and s: *sinister*).

is possible *in vitro*. However, this mechanism can hardly explain how the preferential production of racemized nornicotine is regulated from (−)-nicotine by the plant. In principle, opening of the pyrrolidine ring during the conversion can only yield completely racemized nornicotine [50% of (+) form and 50% of (−) form], unless an asymmetric molecule is involved in some way which could result in more of one enantiomer than the other. This asymmetric molecule is likely to be an enzyme, as already presumed by Leete and Chedekel [4]. Coincidentally, the enzyme(s) catalysing nicotine *N*-demethylation has recently been demonstrated in the tobacco plant [15] and in tobacco cell cultures in the authors' laboratory [16].

The studies presented here show that cell suspension cultures of *N. tabacum* L cv. Wisconsin-38 can convert added (−)-nicotine to (−)-nornicotine exclusively. This reaction appears to be enzymatically enantioselective and the mechanism may be different from that in the intact plant. Although oxidative demethylation may involve the disappearance of the methyl group in the pyrrolidine ring, the possibility of transmethylation cannot be eliminated, since it was observed that bioconversion did take place in homogenates of tobacco plant containing glycine and ethanolamine [17]. It was also presumed that both de- and transmethylation might be involved in the reaction [18]. In view of these possibilities, further investigation, e.g. using (methyl-^{14}C)-nicotine to trace the fate of the C1 unit may reveal the nature of the reaction.

EXPERIMENTAL

Plant material and general. Suspension cultures of *Nicotiana tabacum* cv. Wisconsin-38, established and optimized in this laboratory, were grown in the dark at 25 ± 2° in B_5 medium [19] supplemented with sucrose (30 gl^{-1}), NAA (0.15 mgl^{-1}), kinetin (0.2 mgl^{-1}) and ascorbic acid (5 mgl^{-1}) at pH 5.6. Subculture was carried out, every 20 days, by transferring aseptically 1 g fr. wt of cells to a 250 ml conical flask containing 50 ml medium and incubating on a rotary-shaker at a speed of 90 rpm. Fr. wt of suspension cells was estimated after filtration under red. pres. through Miracloth (CalBiochem). All experimental data presented in this paper are either the means of three replicates ± s.d. or the means of three replicates.

Extraction of alkaloids. Suspended cells were separated from the medium by vacuum filtration. The medium was hydrolysed with 2M KOH at 90° for 1 hr (for prepn of samples which were subject to optical determination, the medium was adjusted to pH 11 with 35% (v/v) NH_4OH) and then extracted ×3 with an equal vol. of $CHCl_3$. After removing the dissolved H_2O with dry Na_2SO_4, the $CHCl_3$ extract was filtered and evaporated to dryness under vacuum. The residue was resuspended in 1 ml of HPLC-grade MeOH, then filtered through a 0.45 μm filter (Whatman) for measurement. The cells were ground up in the same vol. of 1 M HCl and the cell debris removed by filtration. The aq. phase was treated in the same way as the medium.

Chromatography. Analytical TLC was performed on Silica Gel 60 and prep. TLC on a fluorescent (254 nm) Silica Gel 60 Å (1 mm layer) with a solvent system of $CHCl_3$–MeOH–20% NH_4OH (60:10:1, v/v). Alkaloids were detected with an iodoplatinate reagent consisting of 10 ml of 5% (w/v) $PtCl_2$, 5 ml 11M HCl and 240 ml of 2% (w/v) KI. For prep. TLC, alkaloids were identified under UV light.

Quantitation of alkaloids was performed on a Gilson-302 HPLC. Alkaloids were separated at room temp. on a 3.9 × 150 mm, 4 μm particle size Nova-Pak C_{18} column (Millipore Waters), with a gradient mobile phase consisting of MeOH and 0.2% (v/v) H_3PO_4–Et_3N buffer (pH 7.5) at a flow rate of 1 ml min^{-1}. Alkaloids were detected at 254 nm.

Determination of the optical property of alkaloids. The optical rotation of the nornicotine converted from added (−)-nicotine by the cultures was determined on a Perkin-Elmer Model 141 polarimeter (589 nm) at 20 ± 5°. A standard 1 cm sample cell was used. An alternative method of examining the optical property of the nornicotine was performed using chiral GC, in which 6 mg nornicotine sample were dissolved in 0.5 ml CH_2Cl_2 and 10 mg of (*lS*)-(−)-camphanic acid chloride

were added. After a 1 hr reaction at room temp. to produce the *N'*-camphanoyl-nornicotine diastereoisomeric amide derivatives, the sample was further concd in a rotary evaporator. Finally, the concentrates were injected into and separated by a capillary GC on a 30 m DB5 (0.53 mm i.d.) wide-bore column, with an oven temp. of 240° (isothermic) and He as the carrier gas at 2 ml min^{-1}.

Radioactive feeding experiment. DL-[Pyrrolidine-2'-^{14}C]-nicotine (NEN, Du Pont) with a sp. act. of 52.7 mCi $mmol^{-1}$ (purity 97.7%) was used throughout. After addition of 0.5 μCi per flask of tracer, the cultures were removed and the alkaloids extracted using the method described above and $CHCl_3$ soluble compounds were separated on TLC following localization by autoradiography. All fractions and the alkaloids spots on TLC were assayed for radioactivity by scintillation counting.

Identification of unknown compounds by GC-MS. GC-MS operating conditions were as follows: HRGC-EI-MS System (KRATOS MS 50TC); fused silica capillary column (50 m × 0.32 cm i.d., CP-Sil 5); temp. programme, 70–100° at 3° min^{-1} and 100–250° at 5° min^{-1}; carrier gas, He (10%); ionization energy, 70 eV; source temp., 210°. All metabolites were identified by reference to their GC retention time and the MS spectra of authentic standards or authentic MS spectra which are presented as follows.

Nicotine. Retention time 4.43 min *m/z* (rel. int.): 162 $[M]^+$ (58), 161 $[M-H]^+$ (45), 133 $[M-CH_3N]^+$ (64), 119 $[M-C_2HN]^+$ (49), 84 $[M-\text{pyridyl}]^+$ (100), 78 $[M-\text{pyrrolidyl}]^+$ (42), essentially the same as lit. data [20].

Nornicotine. Retention time 5.95 min. *m/z* (rel. int.): 148 $[M]^+$ (50), 147 $[M-H]^+$ (64), 120 $[M-C_2H_4]^+$ (64), 119 $[M-CH_3N]^+$ (100), 118 $[M-C_2H_6]^+$ (53), 105 $[M-C_2H_5N]^+$ (38), 80 $[\text{pyridine}+H]^+$ (52), 70 $[M-\text{pyridyl}]^+$, (94), 41 (48), essentially the same as lit. data [20].

Formylnornicotine. (data from pers. commun.) Retention time 13.1 min. *m/z* (rel. int.): 176 $[M]^+$ (100), 158 (19), 147 $[M-CHO]^+$ (99) 130 (10), 119 (62), 105 (40), 98 $[M-\text{pyridyl}]^+$ (44), 78 $[\text{pyridyl}]^+$, (24), 70 (51).

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