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Stereoselectivity of the demethylation of nicotine piperidine homologues by *Nicotiana plumbaginifolia* cell suspension cultures

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Abstract

The metabolism of (*R*,*S*)-*N*-methylanabasine and (*R*,*S*)-*N*-methylanatabine has been studied in a cell suspension culture of *Nicotiana plumbaginifolia*. Both substrates are effectively demethylated, anabasine or anatabine, respectively, accumulating in the medium. Similarly, there is strong stereoselectivity for the (*R*)-isomers of both substrates. The kinetics of metabolism of (*R*,*S*)-*N*-methylanabasine differ significantly from those of nicotine in that no further degradation of the initial demethylation product occurs. (*R*,*S*)-*N*-Methylanatabine, however, shows kinetics closer to those of nicotine, with loss of alkaloid from the system. Furthermore, (*R*,*S*)-*N*-methylanabasine does not diminish (*S*)-nicotine demethylation, indicating a lack of competition. However, the metabolism of (*S*)-nicotine is affected by the presence of (*R*,*S*)-*N*-methylanabasine. Hence, the demethylation of the piperidine homologues of nicotine is seen to be similar but not identical to that of the pyridine analogues. The implications of these different metabolic profiles in relation to the demethylation activity are discussed.

Keywords: *Nicotiana plumbaginifolia*; (*R*,*S*)-*N*-Methylanabasine; (*R*,*S*)-*N*-Methylanatabine; (*S*)-Nicotine; Demethylation; Cell suspension culture; Chiral selectivity

1. Introduction

The principal alkaloid found in most *Nicotiana* species is (*S*)-nicotine (Fig. 1), the chiral centre at C2′ being established during the condensation of *N*-methylpyrrolinium ion with 'activated' nicotinic acid. It is usually accompanied by smaller amounts of other alkaloids (Leete, 1983; Saitoh et al., 1985). While some of these – such as nornicotine, cotinine and myosmine – are biosynthetically derived from nicotine, the extent to which the configuration at the C2′ is conserved is variable (Armstrong et al., 1999; Leete, 1983; Mesnard et al., 2001). In a few species, such as *Nicotiana alata* and *N. otophora*, nornicotine can accumulate in roots to levels as high as, or even higher than, nicotine.

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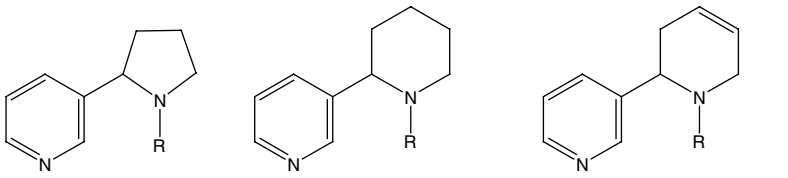

R = CH_3 Nicotine
R = H Nornicotine

R = CH_3 *N*-Methylanabasine
R = H Anabasine

R = CH_3 *N*-Methylanatabine
R = H Anatabine

Fig. 1. Structures of the alkaloids.

These alkaloids are frequently accompanied by variable amounts of other minor components – notably anabasine and anatabine – which are produced by related biosynthetic pathways (Bush et al., 1999; Robins, 1998). In some species, such as *N. glauca*, anabasine is the major alkaloid found in all tissues (Saitoh et al., 1985). While superficially similar to nicotine, these alkaloids differ in three aspects: the presence of a piperidine ring in place of the pyrrolidine ring; the total absence of any *N*-methyl derivatives; and their tendency to be found as mixtures of the (*S*)- and (*R*)-isomers (Armstrong et al., 1999).

Both biochemical and chemical evidence have shown that nornicotine is produced by the demethylation of nicotine (Bush et al., 1999; Leete, 1983), not by a parallel pathway from putrescine via the pyrrolinium ion. In contrast, all the evidence is that anabasine and anatabine are not synthesised via an *N*-methylated intermediate (Fecker et al., 1993; Robins, 1998). The reactions responsible for the *N*-demethylation of nicotine to nornicotine have attracted interest both for their biochemical elucidation and for their potential economic exploitation. It is an important reaction, and it is primarily due to the efficient demethylation of nicotine by hepatic detoxifying systems that the extreme toxicity of this compound to man is not more evident (Gorrod and Schepers, 1999). Nevertheless, nicotine derivatives are showing an increasing potential in treating neurological disorders (Brennan, 2000). Mammalian detoxification involves the intervention of a P-450 oxidase to activate the pyrrolinium ring at the C5′ position (Carlson et al., 1995).

In contrast, the mechanism of this process in plants is still not well understood. It has been established that the C5′ position is not involved in nicotine oxidation in plants (Botte et al., 1997), indicating that the mechanism of degradation is not equivalent. It is also apparent that demethylation can be effectively carried out on a wide range of compounds modified either in the heterocycle or in the *N*-substituent (Boswell et al., 1993, 1999; Dawson, 1951; Leete, 1983; Mesnard et al., 2001).

As part of a programme aimed at establishing the mechanism of nicotine demethylation in plants, the chemio- and stereo-specificity of the reaction in terms of the substrates that can be demethylated is being examined in cell suspension cultures of *N. plumbaginifolia*, which have previously proved to be efficient at converting exogenous nicotine to nornicotine (Manceau et al., 1989). In order to evaluate the involvement of each cycle in the reaction and the degree of selectivity of the reaction, three types of nicotine analogue have been synthesised. In the first type, modification was made to the N-substituent. This showed that the demethylation activity was limited to only small increases in the size of the substituent group present (Boswell et al., 1999). In the second type, modification was made to the pyridine ring, notably the replacement of the heterocycle with a phenyl group. These analogues were efficiently metabolised and competed with nicotine for the demethylation activity (Mesnard et al., 2001). It was shown that both isomers in (*R*,*S*)-nicotine or pyridine analogues were demethylated, but at markedly different rates (Mesnard et al., 2001) indicating a degree of chiral selectivity in the process.

In this paper, we report the demethylation of the third type of modification, in which the pyrrolidine cycle has been replaced by a piperidine ring. (*R*,*S*)-*N*-Methylanatabine and (*R*,*S*)-*N*-methylanabasine, the non-natural *N*-methyl derivatives of anatabine and anabasine, have been synthesised (Felpin et al., 2001) and fed to cell suspension cultures of *N. plumbaginifolia*. This paper reports the metabolism of these compounds.

2. Results and discussion

2.1. Metabolism of (R,S)-N-methylanabasine or(R,S)-N-methylanatabine

The metabolism in cell cultures of *N. plumbaginifolia* of (*R*,*S*)-*N*-methylanabasine and (*R*,*S*)-*N*-methylanatabine was followed by HPLC in an initial experiment over 10 days. In order to confirm the stability of these products under biotransformation conditions, either 7.1 μmol of (*R*,*S*)*N*-methylanabasine or 6.2 μmol of (*R*,*S*)*N*-methylanatabine was incubated in FMS medium without cells. No degradation of either substrate occurred (data not shown).

In contrast, incubation of 7.1 μmol of (*R*,*S*)*N*-methylanabasine with 2.0 g FW of *N. plumbaginifolia*

cells in non-growth-stimulating conditions led to the demethylation of approximately 50% of the (*R*,*S*)-*N*-methylanabasine within 4 days (Fig. 2(a)). Within 2 days, both *N*-methylanabasine and anabasine were detected in the cells at about 0.2 μmol (Fig. 2(b)). Thereafter, the intracellular concentration of *N*-methylanabasine remained constant, while the level of the anabasine steadily increased, reaching 0.5 μmol by day 10. Cells remained healthy throughout this time course. This is a similar profile to that observed with nicotine (Mesnard et al., 2001), indicating that demethylation was almost certainly intracellular, *N*-methylanabasine being taken up from the medium and anabasine being released. Such a proposal is supported by the steady accumulation of anabasine in the medium (Fig. 2(a)), reaching 3.3 μmol (40%) at 4 days and slowly increasing up to 3.5 μmol at 8 days. At this point, 49% of the substrate was unmetabolised and 49% was present as the product of demethylation. No other product of metabolism was detected.

Similarly, when 6.2 μmol of (*R*,*S*)-*N*-methylanatabine was incubated with 2.0 g FW of *N. plumbaginifolia* cells in non-growth-stimulating conditions, 62% of the (*R*,*S*)*N*-methylanatabine was degraded within 4 days (Fig. 2(c)). Metabolism again appeared to be intracellular, as shown in Fig. 2(d), and the product of demethylation, anatabine, was the only compound seen to accumulate. Within 2 days, the intracellular substrate concentration reached a maximum (0.6 μmol), while the product maximal (0.5 μmol) was approximately 2 days later (Fig. 2(d)). Thereafter, both substrate and product could be detected in the cells in steadily decreasing amounts, reaching zero around 10 days. Cells remained healthy throughout this time course. This profile differs slightly from those of (*S*)-nicotine and (*R*,*S*)-*N*-methylanabasine, which both remained elevated in the cells at day 10. In parallel, anatabine accumulated in the medium, but only about 0.6 μmol (approximately 11%) had accumulated at 4 days (Fig. 2(c)). Thus, in contrast to the degradation of (*R*,*S*)-*N*-methylanabasine, about 40% of the compound was not detected at 4 days in either substrate or product of demethylation, a profile closer to that for nicotine metabolism than that for (*R*,*S*)-*N*-methylanabasine metabolism (Fig. 2(a) and (b)). Thereafter, however, the amount of anatabine accumulated steadily, such that by 8 days, 2.0 μmol (39%) of demethylation product and 1.9 μmol (37%) of substrate remained, a total recovery of about 76%.

Thus, efficient demethylation of both (*R*,*S*)-*N*-methylanatabine and (*R*,*S*)-*N*-methylanabasine occurs, as seen for nicotine and analogues altered in the pyridine moiety (Mesnard et al., 2001). The profiles of degradation differ slightly, in that (*R*,*S*)-*N*-methylanatabine does not persist in the cells and, by 8 days, about 25% of the initial compound present in substrate is not recovered in product. This profile is closer to that of nicotine degradation than that of (*R*,*S*)-*N*-methylanabasine in that net loss of alkaloid has occurred. However, no minor products that could represent either further metabolism of

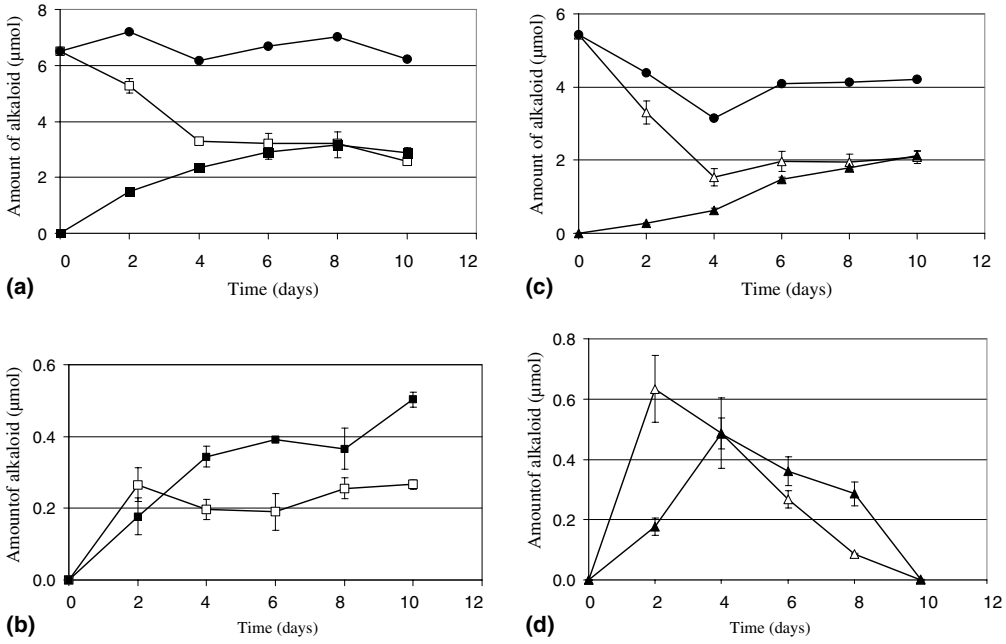

Fig. 2. Metabolism of (a and b) (*R*,*S*)-*N*-methylanabasine or (c and d) (*R*,*S*)-*N*-methylanatabine by cell cultures of *N. plumbaginifolia*. (a) and (c) show the alkaloid content of the medium, (b) and (d) the alkaloid content of the cells. Each point represents the mean ± s.d. for 2 cultures and at least 3 determinations by HPLC. (□) (*R*,*S*)-*N*-methylanabasine, (■) (*R*,*S*)-anabasine, (△) (*R*,*S*)-*N*-methylanatabine, (▲) (*R*,*S*)-anatabine, (●) total alkaloid ($substrate_{cells} + substrate_{medium} + product_{cells} + product_{medium}$).

anatabine or alternative metabolites of *N*-methylanatabine were identified. For the demethylation of *N*-methylanabasine, only anabasine was detected as product and no decrease was observed in the total (substrate + product) during the middle period of the culture.

2.2. Chiral selectivity of demethylation of (*R,S*)-*N*-methylanabasine and (*R,S*)-*N*-methylanatabine

A simple explanation of the demethylation profiles observed by HPLC is that the demethylation activity is not equivalent for the (*S*)- and (*R*)-isomers. The chirality of the residual substrates and the demethylation products was therefore followed in duplicate experiments by chiral GC.

The profiles of the residual (*R*)- and (*S*)-*N*-methylanabasine and of the (*R*)- and (*S*)-anabasine accumulated in the medium are presented in Fig. 3(a) and (b). It is seen that, as with (*R*,*S*)-nicotine, the (*R*)-isomer was very much more rapidly demethylated than the (*S*)-isomer (Fig. 3(a)), leading to a rapid accumulation of (*R*)-anabasine, which was accompanied by a slower accumulation of (*S*)-anabasine, to about 15% of the (*R*)-isomer (Fig. 3(b)). When compared with (*R*,*S*)-nicotine (Mesnard et al., 2001), (*R*)-*N*-methylanabasine was more rapidly lost than (*R*)-nicotine, essentially no (*R*)-*N*-methylanabasine remaining in the medium after 4 days. This observation is open to two interpretations: either only the (*R*)-isomer is transported into the cells or the (*R*)-isomer is a very much better substrate for the *N*-demethylating activity. When the intracellular pool was analysed, it was seen to contain both isomers of (*R*,*S*)-*N*-methylanabasine, with the (*S*)-isomer in excess of the (*R*) (Fig. 4). This is compatible with both isomers being taken up by the cells but the (*R*)-isomer being more rapidly demethylated, the (*R*)-anabasine being excreted and accumulating in the medium. It was not possible to obtain a reliable determination of the isomeric composition of the low intracellular levels of (*R*)- and (*S*)-anabasine.

A very similar profile was seen for the selectivity of demethylation of the two isomers of (*R*,*S*)-*N*-methylanatabine (Fig. 3(c) and (d)). Again, it was the (*R*)-isomer of (*R*,*S*)-*N*-methylanatabine that rapidly disappeared from the medium and (*R*)-anatabine that accumulated. The (*S*)-isomer also underwent demethylation, but more slowly, and (*S*)-anatabine accumulated in the medium to only ca. 35% of the level of the (*R*)-isomer. The medium content of (*R*)-*N*-methylanatabine was essentially reduced to zero within 4 days. While the overall profile of the chirality of demethylation is very close to that of (*R*,*S*)-*N*-methylanabasine, there was ca. 25% loss of alkaloid from the system (Fig. 2(c) and (d)). However, it cannot be ascertained whether this loss is of only one isomer or whether both isomers of anatabine are subject to further metabolism. What is apparent is that, as with (*R*,*S*)-nicotine, the rate of demethylation for the two isomers was not equivalent and that the (*R*)-isomer was degraded preferentially (Mesnard et al., 2001).

Indeed, for both (*R*,*S*)-*N*-methylanabasine and (*R*,*S*)-*N*-methylanatabine, it cannot be definitively concluded whether the (*S*)-isomer of the product is derived from

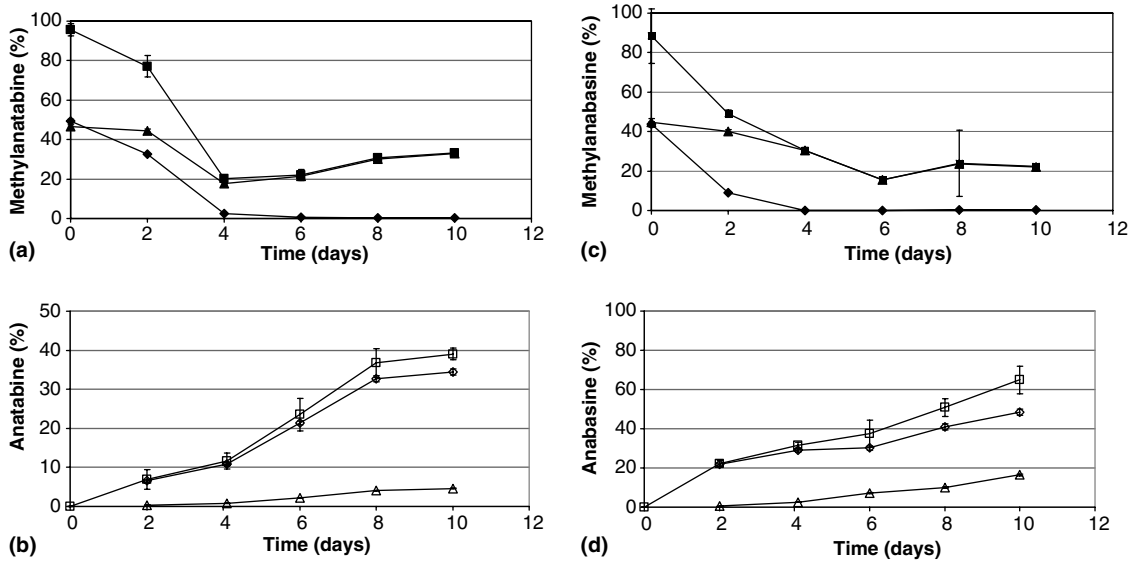

Fig. 3. Metabolism of (a and b) (*R*,*S*)-*N*-methylanabasine or (c and d) (*R*,*S*)-*N*-methylanatabine by cell cultures of *N. plumbaginifolia* indicating the amount of (*R*)- and (*S*)-isomer present in the medium for each compound. (a) and (c) show the substrate content, (b) and (d) the product content. Each point represents the mean ± s.d. for 2 cultures and at least 2 determinations by GC. The total quantity of alkaloid was determined by GC and the proportion of each isomer present by chiral GC. For substrates (a and c), (■) total, (◆) (*R*)-isomer, (▲) (*S*)-isomer; for products (b and d), (□) total, (◇) (*R*)-isomer, (△) (*S*)-isomer.

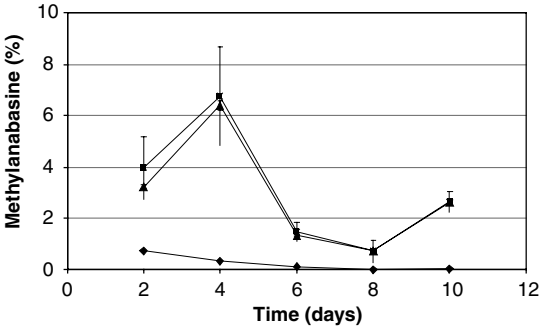

Fig. 4. Metabolism of (*R*,*S*)-*N*-methylanabasine by cell cultures of *N. plumbaginifolia* indicating the amount of (*R*)- and (*S*)-isomer present in the cells. Each point represents the mean ± s.d. for at least 2 determinations by GC. The total quantity of alkaloid was determined by GC and the proportion of each isomer by chiral GC. (■) total, (◆) (*R*)-isomer, (▲) (*S*)-isomer.

the (*S*)-isomer of the substrate or whether some racemisation has occurred. From the data presented in Fig. 3(a) and (c), it would appear that, from day 4 onwards, the residual alkaloids in the medium are composed of approximately 100% of the (*S*)-isomer. However, the (*S*)-isomers of both products accumulate steadily over days 4–10 (Fig. 3(b) and (d)). Hence, it would appear that either the cells contain a racemase, slowly converting the (*R*)- to the (*S*)-isomer, or the demethylase does not retain enantiomeric purity at 100%. In view of the fact that both anatabine and anabasine naturally accumulate as enantiomeric mixtures (Armstrong et al., 1999), the presence of a racemase is likely. Nicotine biosynthesis produces exclusively the (*S*)-isomer (Leete, 1983), although whether such rigid stereoselectivity also occurs for anatabine and anabasine biosynthesis is not known. However, both isomers of nornicotine have been reported, consistent with racemase activity on the *N*-demethylated product.

2.3. Co-feeding with (*S*)-nicotine and (*R,S*)-*N*-methylanabasine

It was previously demonstrated that demethylation by *N. plumbaginifolia* of nicotine and analogues in the pyridine moiety showed competition (Mesnard et al., 2001). Experiments were therefore conducted with (*R*,*S*)-*N*-methylanabasine, homologue in the pyrrolinium moiety, to see whether this modification had a similar effect.

Cell cultures supplied with nicotine alone showed essentially the same profile of nicotine catabolism as seen previously, in that nicotine was consumed continuously and nornicotine accumulated (Mesnard et al., 2001). Within 8 days, for 6.2 μmol of nicotine consumed, ca. 4 μmol of nornicotine accumulated (Fig. 5(a)). Thus, only 71% of the initial alkaloid was

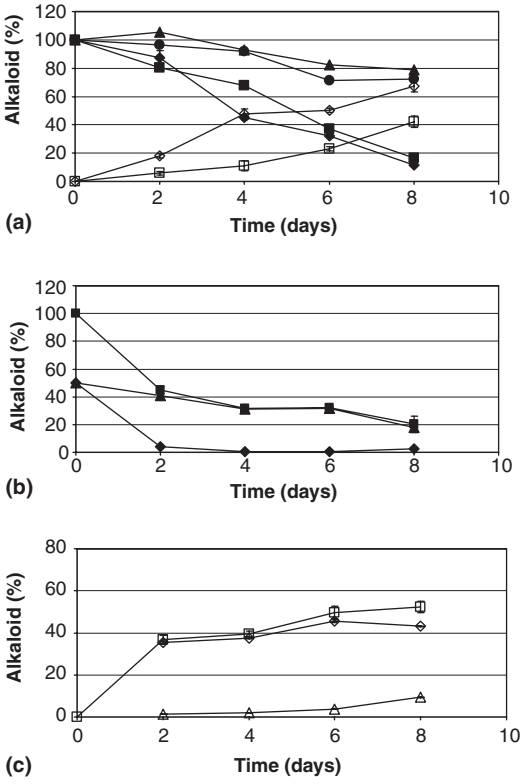

Fig. 5. Metabolism of (*S*)-nicotine and (*R*,*S*)-*N*-methylanabasine simultaneously by cell cultures of *N. plumbaginifolia*. (a) The (*S*)-nicotine, nornicotine and total alkaloid content in the medium in the presence (◆, ◇, ▲), respectively) or absence (■, □, ●, respectively) of (*R*,*S*)-*N*-methylanabasine; (b) and (c) the *N*-methylanabasine (filled symbols) and anabasine (open symbols) content in the medium in the presence of (*S*)-nicotine. (◆, ◇) (*R*)-isomer, (▲, △) (*S*)-isomer, (■, □) total. Each point represents the mean ± s.d. for 2 cultures and at least 3 determinations by HPLC or GC.

present at day 8, consistent with previous data indicating that nornicotine shows substantial further catabolism.

When exhibited simultaneously, both (*S*)-nicotine and (*R*,*S*)-*N*-methylanabasine were taken into the cells and demethylated (Fig. 5). The intracellular concentrations of nicotine and *N*-methylanabasine and their demethylation products were similar to when either alkaloid was supplied alone (data not shown). Both products of demethylation, nornicotine and anabasine, were seen to accumulate steadily in the medium. The presence of (*R*,*S*)-*N*-methylanabasine had no apparent effect on the rate of metabolism of (*S*)-nicotine, although the extent to which nornicotine accumulated was enhanced. Whether this is an influence on the further metabolism of nornicotine, leading to higher accumulation, needs further investigation. However, the overall loss of the nicotine alkaloid series from the system occurred to an equivalent level both in the presence and absence of (*R*,*S*)-*N*-methylanabasine (Fig. 5(a)).

Furthermore, the presence of (*S*)-nicotine had no apparent effect on the demethylation of (*R*,*S*)-*N*-methylanabasine (compare Figs. 3 and 5): neither the rate of demethylation nor the stereoselectivity of the reaction was affected. In addition, the total alkaloid for the anabasine series remained essentially unaltered, as for (*R*,*S*)-*N*-methylanabasine alone.

Thus, neither the rate of anabasine accumulation nor the stereoselectivity of the demethylation of (*R*,*S*)-*N*-methylanabasine was influenced by the presence of nicotine, nor was the metabolism of nicotine in the presence of *N*-methylanabasine markedly different to that for nicotine alone.

Hence, the co-presence of the two substrates does not appear to have mutually influenced their demethylation, the kinetics of degradation not being modified. The only notable effect is that of enhanced nornicotine accumulation. This indicates that, in contrast to the situation of substrates modified on the pyridine ring of nicotine (Mesnard et al., 2001), demethylation activity is sensitive to the size and/or configuration of the ring containing the *N*-methyl group against which the activity is directed.

3. Conclusions

It has previously been shown that *Nicotiana* spp. cell cultures are active in demethylating a range of substrates (Boswell et al., 1999; Dawson, 1951; Leete, 1983; Manceau et al., 1989; Mesnard et al., 2001), including those with *N*-methylation or *N*-ethylation on the piperidine ring of C_6 homologues of nicotine (Dawson, 1951). It is now confirmed that this demethylation is intracellular, although, as frequently observed with in vitro cultures, the majority of the product accumulates in the medium. This is to be expected in that what little evidence is available indicates that demethylation in *Nicotiana* probably involves a P-450 oxidase in the microsomal fraction (Chelvarajan et al., 1993) or a mixed function oxidase (Hao and Yeoman, 1998). Either case will have a requirement for reduced pyridine cofactor. Furthermore, it is apparent that the methyl group can be transferred to the tetrahydrofolate pool (Mesnard et al., 2002).

The two homologues tested show slightly different profiles of demethylation, in that the total (substrate + product) remained close to 100% for (*R*,*S*)-*N*-methylanabasine throughout the analytical period, whereas for (*R*,*S*)-*N*-methylanatabine 30–40% of the alkaloid was undetected, depending on the extent of advancement of the reaction. It would appear that, while efficient at demethylating both these compounds, the cell cultures do not possess the capacity to degrade further anabasine, but that anatabine may be subject to metabolism. However, for neither series of compounds was any other metabolite identified. In its profile, the demethylation of (*R*,*S*)-*N*-methylanatabine is thus closer to that of nicotine than that of (*R*,*S*)-*N*-methylanabasine.

However, both substrates show the same stereoselectivity of demethylation as (*R*,*S*)-nicotine, in that the (*R*)-isomer is strongly preferred to the (*S*)-isomer. Thus, over the time-course of the experiment, the culture medium is relatively enriched in (*S*)-*N*-methylanabasine or (*S*)-*N*-methylanatabine, while (*R*)-anabasine and (*R*)-anatabine, respectively, accumulate. In this accumulation of the products of demethylation, the metabolism of these substrates differs very markedly from that of nicotine, in that (*R*)-nornicotine is rapidly further degraded. While it has been suggested that the degradation probably is initiated with a ring-opening reaction, as described for the degradation of nicotine by various micro-organisms (Kaiser et al., 1996), this has not yet been experimentally confirmed.

With (*S*)-nicotine, it is increasingly evident that demethylation proceeds via oxidation of the *N*-methyl group, without involving the ring (Botte et al., 1997; Molinié, 2005), in contrast to the proposed mechanism for the oxidation of (*S*)-nicotine by liver P450 oxidase (Carlson et al., 1995). It is now shown that the demethylation activity present in *N. plumbaginifolia* can also act on non-natural *N*-methylpiperidine homologues of nicotine. The stereoselectivity of demethylation of (*R*,*S*)-*N*-methylanatabine and (*R*,*S*)-*N*-methylanabasine is the same as found for nicotine (Mesnard et al., 2001), in that the (*R*)-isomer is preferentially degraded, which could imply that the same enzyme system is responsible for the demethylation of all these alkaloids. However, *N*-methylanabasine does not apparently compete with nicotine for demethylation activity, suggesting the possibility that more than one demethylation activity might be present. Further study of these demethylation processes is required to resolve these discrepancies.

4. Experimental

4.1. Materials

(*R*,*S*)-*N*-Methylanabasine, (*R*,*S*)-*N*-methylanatabine (*R*,*S*)-anabasine and (*R*,*S*)-anatabine were synthesised as described previously (Felpin et al., 2001). An authentic sample of synthetic (*S*)-(−)-anabasine (Amat et al., 2002) was obtained from Pr. M. Amat (University of Barcelona, Barcelona, Spain). A non-racemic mixture of (*R*,*S*)-anabasine ((*R*):(*S*) approximately 4:1) was obtained by extracting Burley tobacco leaf, as described previously (Armstrong et al., 1999).

4.2. Cell suspension cultures

A stable culture of green *N. plumbaginifolia* was cultured on medium 'FMD' as described previously (Mesnard et al., 2001). Essentially, this medium is a modified 'LS' medium (Linsmaier and Skoog, 1965) and contains sucrose (87.6 mM), 2,4-dichlorophenoxyacetic acid (0.9 μM) and 6-benzylaminopurine (0.4 μM) to promote growth.

4.3. Protocol for bioconversion assays

Cells grown in culture medium 'FMD' were harvested aseptically during the exponential growth phase (day 8 of the culture cycle) and transferred into a non-growth-stimulating medium 'FMS' (Mesnard et al., 2001). This minimal medium contains the macro-elements of medium 'FMD' but lacks micro-elements, vitamins, sucrose and phytohormones. Very limited growth occurs under these conditions. Biotransformation was conducted as described previously (Mesnard et al., 2001) in 'FMS' medium supplemented with nicotine (15.4 μmol), *N*-methylanabasine (7.1 μmol) or *N*-methylanatabine (6.2 μmol). Competition experiments were conducted under the same conditions using nicotine (7.7 μmol) with *N*-methylanabasine (3.6 μmol). All experiments were carried out at least twice, with duplicate analyses made in each experiment.

4.4. Extraction procedures

Cells were separated from medium by filtration. Cells were freeze-dried and medium stored at −20 °C until required. Alkaloids were recovered from freeze-dried cells by extraction with 25 ml $CHCl_3$–NH_4OH 30% (24/1 v/v) twice under reflux for 2 h. After filtration, the pooled extract was dried under reduced pressure (33 °C).

For HPLC, cellular extracts were dissolved in mobile phase (1–5 ml) and filtered. Culture media were diluted as required in mobile phase, filtered and directly injected onto the HPLC system.

For chiral GC, a portion of the cellular extract in mobile phase was rigorously extracted with $CHCl_3$, the organic phases pooled and dried, and the residue taken into MeOH. Alkaloids were recovered from culture media by solid phase extraction chromatography. Following basification to pH > 10 (NH_4^+ solution), this solution was applied to a preconditioned (washed with 5 ml MeOH and 5 ml 1% NH_4^+ solution) solid phase extraction cartridge (500 mg Discovery C18, Supelco, www.sigmaaldrich.com) fitted to a Visiprep vacuum manifold (Supelco). Sample was introduced by low suction, the cartridge rinsed with 1% NH_4^+ solution (5 ml) and dried with a flow of N_2 gas for 60 min. Compounds were recovered in methanol (10 ml) and the volume reduced to approximately 0.5 ml.

4.5. HPLC analysis (quantitative)

Following optimisation of the conditions, alkaloids were separated on an HPLC system composed of a Merck 6200 pump with a Merck 4250 UV detector in the following conditions: column, C18-Nucleosyl (250 × 4.6 mm, Macherey Nagel, France); eluant, per litre: 5 ml CH_3CN, 4 ml THF, 0.5 ml H_3PO_4 (85%), 1 g KH_2PO_4, 10 mg NaN_3, 0.5 ml triethylamine (pH 3.5 with KOH); detector, 260 nm; flow, isocratic at 0.8 ml/min. Each sample was analysed three times. In these conditions, satisfactory resolution of all target alkaloids was obtained. Detection limits were 10 ng for nicotine and nornicotine, 25 ng for *N*-methylanabasine and anabasine and 25 and 50 ng for *N*-methylanatabine and anatabine, respectively. For all alkaloids, calibration was linear to at least 100 ng.

4.6. GC analysis (quantitative)

Quantification of alkaloids was performed by GC on a Hewlett Packard 6890N (www.hp.com) chromatograph fitted with a Hewlett Packard 6783 injector; carrier gas, helium at 1.2 ml/min (constant flow); split ratio, 1:20; column, fused poly(5% diphenyl/95% dimethylsiloxane) deactivated for non-specific interaction with basic compounds (PTA-5, Supelco, 30 m, i.d. 0.32 mm, film thickness, 0.52 μm), injector temperature, 220 °C, detection by FID at 280 °C; injected volume (manual), 1 μl. Elution was by thermal gradient: 80 °C for 1 min; 10 °C/min to 175 °C, 6 min at 175 °C, 40 °C/min to 260 °C, 1 min at 260 °C. Calibration was by reference to external standards (range 0.05–0.5 mg/ml; nicotine $r^2 = 0.9979$, nornicotine $r^2 = 0.9994$, *N*-methylanabasine $r^2 = 0.9981$, anabasine $r^2 = 0.9990$ *N*-methylanatabine $r^2 = 0.9991$, anatabine $r^2 = 0.9995$) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.5 mg/ml) was used as internal correction reference. The identity of the eluted products was confirmed by co-elution with authentic standards and by GC-MS.

4.7. GC analysis (chiral)

Alkaloid stereoisomers were separated by GC on a Hewlett Packard 6510 (www.hp.com) chromatograph; carrier gas, hydrogen at 1.0 ml/min (constant flow); split ratio, 1:10; column, BETA CHIRODEX (Supelco, 30 m, i.d. 0.32 mm, film thickness, 0.52 μm), injector temperature, 220 °C, detection by FID at 250 °C; injected volume (manual), 1 μl. Elution was by thermal gradient. For *N*-methylanatabine and anatabine: 100 °C for 1 min; 20 °C/min to 150 °C, 6 min at 150 °C, 20 °C/min to 170 °C, 6 min at 170 °C, 20 °C/min to 210 °C, 1 min at 210 °C. For *N*-methylanabasine: 100 °C for 1 min; 10 °C/min to 180 °C, 20 °C/min to 210 °C, 2 min at 210 °C. Under these conditions,

(*R*)- and (*S*)-anabasine were not resolved. (*R*)- and (*S*)-anabasine were therefore determined as their trifluoroacetyl derivatives (Armstrong et al., 1999) under the same chromatographic conditions with the following thermal gradient: 100 °C for 1 min; 5 °C/min to 210 °C, 1 min at 210 °C.

Peak assignment was by reference to external standards: (*R*)- and (*S*)-anatabine on the ratio present in an extract of Burley tobacco leaf, previously shown to contain (*R*):(*S*)-anatabine in a ratio of 4:1 (Armstrong et al., 1999); (*R*)- and (*S*)-anabasine by reference to an authentic synthetic sample of (*S*)-(−)-anabasine (Amat et al., 2002).

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