Biosynthesis of Anabasine in Transformed Root Cultures of *Nicotiana* species

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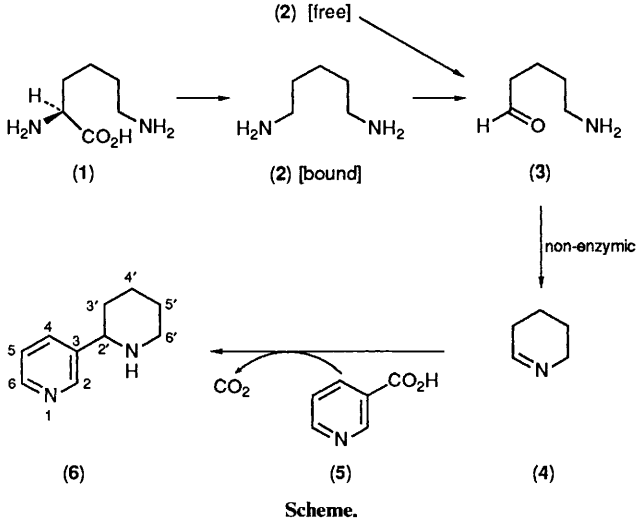
The biosynthesis of 3-(2-piperidyl)pyridine (anabasine) (**6**) has been studied in transformed root cultures of *Nicotiana rustica* and *N. tabacum* using cadaverine precursors selectively labelled with deuterium. The labelling pattern in anabasine (**6**) derived biosynthetically from [2,2,4,4-$^2H_4$]-cadaverine in *N. tabacum* could not be established by ^2H NMR spectroscopy because of the closeness of the chemical shifts of the labelled ^2H atoms. However, the complete labelling pattern for the tetradeuteriated anabasine (**10**) was established by ^1H NMR spectroscopy, because of the very high incorporation of $^2H_4$ species (dilution in anabasine *ca.* 1). Two AB systems were observed for the protons at C-4′ and C-6′, and a singlet for the C-2′ proton in anabasine (**10**). No deuterium was incorporated into the nicotinic acid portion of anabasine. The labelling patterns in anabasine derived biosynthetically from (*S*)- and (*R*)-[1-^2H]cadaverine in *N. rustica* were determined by ^2H NMR spectroscopy. These labelling patterns, due to mixtures of species (**11**) and (**12**) and (**13**)–(**16**), respectively, show that the *pro-R* hydrogen of cadaverine is retained and the *pro-S* hydrogen is lost at the position which becomes C-2′ of anabasine. In addition, the labelling patterns are consistent with formation of equal amounts of (*R*)- and (*S*)-anabasine.

Anabasine (**6**) was first isolated from *Anabasis aphylla* (fam. Chenopodiaceae) where it has the (*S*)-(−)-absolute configuration.[1] It is also present in many *Nicotiana* species (Solanaceae), *e.g.*, it is the main constituent of *N. glauca* (tree tobacco), where it is almost racemic. A range of optical rotations [(+) and (−)] have been reported for anabasine isolated from different plant species.[2]

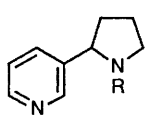
The piperidine ring of anabasine (**6**) is derived from L-lysine (**1**) (see Scheme). In *Nicotiana*, biosynthesis cannot proceed



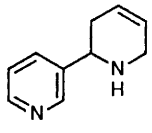
Scheme.

via a symmetrical intermediate [free cadaverine (**2**)], because feeding [2-^{14}C]lysine to *N. glauca* was found to give anabasine with all the radioactivity at the C-2′ position.[3] On the other hand, biosynthesis is unlikely to occur *via* a series of *N*-methylated intermediates commencing with ε-*N*-methyl-lysine, with eventual demethylation of *N*-methylanabasine, since when *N*-methylanabasine was isolated from *Nicotiana* plants which had been fed with [2-^{14}C]lysine[4] or with [*methyl*-^{14}C]methionine,[5] it was found to be unlabelled, in spite of the incorporation into anabasine of label from lysine. The biosynthesis of anabasine (**6**) therefore differs from that of nicotine (**7**) and nornicotine (**8**), which proceeds *via* a free symmetrical diamine (putrescine) which is subsequently *N*-methylated.

Although free cadaverine (**2**) cannot be an intermediate in anabasine (**6**) biosynthesis from lysine (**1**), it is nevertheless incorporated well into the piperidine ring of the alkaloid.[6] Cadaverine has been shown to stimulate the production of anabasine in 'hairy root' cultures of *Nicotiana* species, obtained following genetic transformation with *Agrobacterium rhizogenes*.[7,8] [These cultures normally produce nicotine (**7**), though smaller quantities of anabasine (**6**), nornicotine (**8**), and anatabine (**9**) are also present.] Furthermore, when unlabelled



(**7**) R = Me
(**8**) R = H



(**9**)

cadaverine was fed together with L-[U-^{14}C]lysine to transformed root cultures of *N. hesperis*, the incorporation of lysine into anabasine was undiminished, even though anabasine production was greatly increased.[7] This suggested a concerted decarboxylation and oxidation of lysine to 5-aminopentanal (**3**) unaffected by the simultaneous utilisation of free cadaverine and was consistent with a suggested mechanism in which the cadaverine derived from lysine might be enzymically bound, therefore being neither free nor symmetrical.[4,5] It was argued[7] that exogenous free cadaverine could be metabolised to anabasine without interfering with the incorporation of labelled lysine provided that this metabolism was rapid in relation to the rate of exchange of free with bound cadaverine.

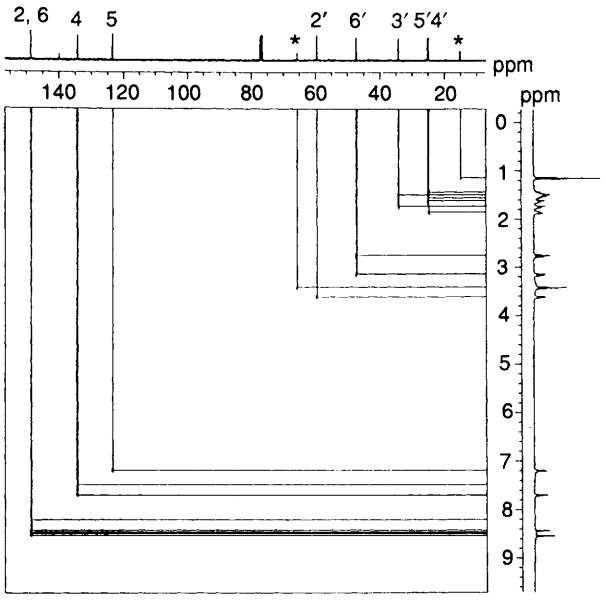


Figure 1. ^{13}C-^{1}H COSY spectrum of anabasine (**6**) in $CDCl_3$ (*, impurity).

Table. ^{13}C and ^{1}H NMR spectroscopic data for anabasine (**6**).

	^{13}C Chemical shifts δ/ppm	^{1}H Chemical shifts δ/ppm
2′	59.6	3.62
3′	34.3	1.52a, 1.75e
4′	25.0	1.45a, 1.86e
5′	25.3	1.49–1.63
6′	47.4	2.76a, 3.15e
2	148.6	8.55
3	140	—
4	134.3	7.71
5	123.4	7.21
6	148.6	8.43

a, axial; e, equatorial.

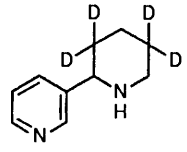
The marked stimulation of anabasine production by cadaverine in *Nicotiana* root cultures,[7,8] however, raised the question of whether the biosynthesis in this case was characteristic of that normally occurring in *Nicotiana* plants, where it has been established that the pyridine ring is derived from nicotinic acid,[9] or whether both rings of anabasine might be generated from cadaverine by an alternative and possibly aberrant mechanism demonstrated in pea and lupin extracts.[10] We here report the use of selectively deuteriated cadaverines to answer this question, to investigate the stereochemistry of the enzymic processes involved in the biosynthesis, and to establish the optical purity of the anabasine produced.

Results and Discussion

Genetic transformation with *Agrobacterium rhizogenes* has produced 'hairy root' cultures of a range of *Nicotiana* species; in general, the relative proportions of the alkaloids they produce is closely correlated with the proportions observed in the roots of intact, untransformed plants.[11] In the presence of cadaverine (**2**), anabasine (**6**) production is stimulated and that of nicotine (**7**) is inhibited; anabasine can account for *ca.* 70% of alkaloid production at 10mM cadaverine.[8] Transformed root cultures of *N. tabacum* (strain SC58) and *N. rustica* were selected for the work described here. These cultures produce mainly nicotine (**7**), together with small amounts of anatabine (**9**), nornicotine (**8**), and anabasine (**6**).

In order to carry out the biosynthetic studies, a detailed assignment of the ^{1}H NMR spectrum of anabasine (**6**) was required. Leete[12] has fed DL-[4,5-$^{13}C_2$]lysine to *Nicotiana glauca* and established the labelling patterns in anabasine by ^{13}C NMR spectroscopy, thus establishing rigorous assignments for the ^{13}C NMR spectrum of anabasine. Use of heteronuclear ^{13}C-^{1}H chemical shift correlation spectroscopy (Figure 1) on anabasine (**6**) in deuteriochloroform gave the assignments for the protons in anabasine (Table). In particular, the 2′-H at δ 3.62 and the 6′-H_2 at δ 2.76 and 3.15 were identified. Homonuclear (^{1}H) chemical shift correlation spectroscopy and NOE difference spectroscopy confirmed the proton assignments and established the chemical shift values for axial and equatorial protons of anabasine, most importantly 6′-H_{ax} at δ 2.76 and 6′-H_{eq} at δ 3.15 (see Table).

To begin the biosynthetic work, [2,2,4,4-$^{2}H_4$]cadaverine dihydrochloride (94% $^{2}H_4$ species) was made by exchanging the α-protons of glutaronitrile with $^{2}H_2O$, followed by reduction and acidification.[13] This material at *ca.* 1mM was fed to six-day old transformed *N. tabacum* cultures and the alkaloid mixture was isolated after 12 d. The alkaloids were identified by high performance liquid chromatography (HPLC)–mass spectroscopy (MS)[8] and by gas liquid chromatography (GLC). The mixture of anabasine (23%), nicotine (64%), anatabine (6%), and a small amount of nornicotine was separated by repeated preparative silica gel TLC. About 1 mg of anabasine was finally obtained. The ^{2}H NMR spectrum of anabasine taken in chloroform showed a broad unresolved band at δ 1.5–1.8, which was not very informative. However, clear evidence for almost complete incorporation of ^{2}H into the 3- and 5-positions of the piperidine ring of anabasine to give the labelling pattern in (**10**) was obtained from the ^{1}H NMR spectrum, due to the absence of



(**10**)

signals for the 3′ and 5′-protons. In addition, changes in the coupling with respect to unlabelled material were observed for the signals due to 2′-H and 6′-H_2 of anabasine. Thus, 2′-H was seen as a broadened singlet instead of a doublet, and 6′-H_{ax} changed from a doublet of doublets to a doublet, *i.e.* the protons at C-6 exhibited a clear AB system with no additional proton coupling. The highfield part of the AB system due to the C-4′ protons was partly obscured by impurities. No incorporation of deuterium was observed in the pyridine ring of anabasine, confirming that this portion of the molecule is not derived from cadaverine.

(*R*)-[1-^{2}H]Cadaverine dihydrochloride (90% $^{2}H_1$ species) was prepared by decarboxylation of L-lysine (**1**) in $^{2}H_2O$ using lysine decarboxylase followed by acidification.[14] the (*S*)-isomer (85% $^{2}H_1$ species) was obtained by similar decarboxylation of the L-component of [2-^{2}H]-DL-lysine in $^{1}H_2O$. These enantiomers have been used by us to study the stereochemistry of the enzymic processes involved in quinolizidine alkaloid biosynthesis.[15] Samples of (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochloride were each fed to transformed root cultures of *Nicotiana rustica* (this culture produces a mixture of alkaloids similar to that from *N. tabacum*) and the anabasine was isolated and separated from the mixture of alkaloids as before. The ^{2}H NMR spectrum obtained in chloroform after feeding with (*S*)-

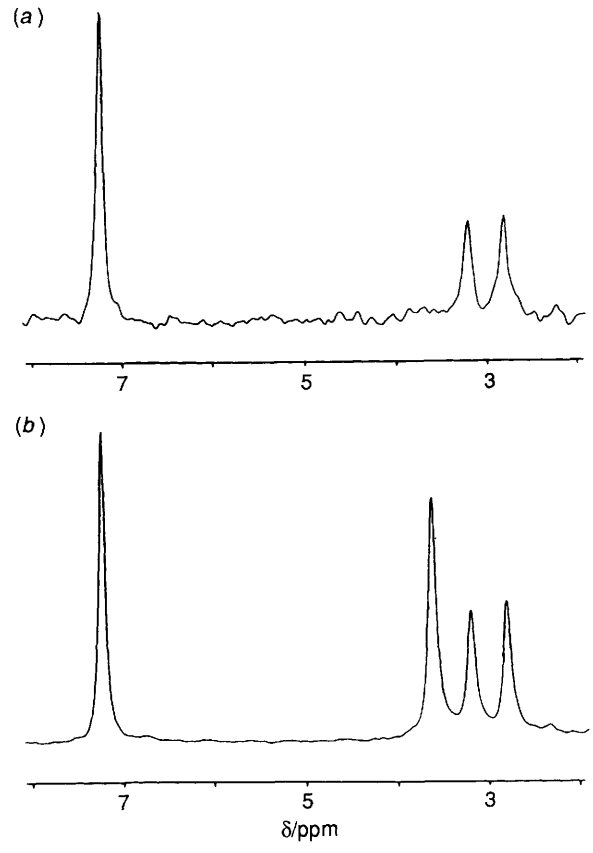
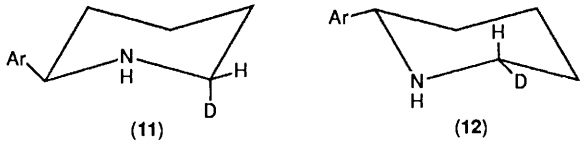


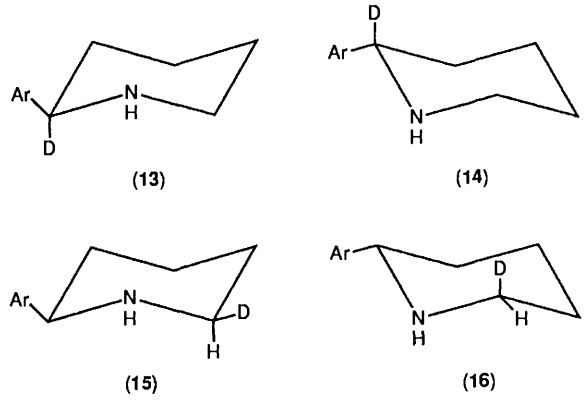
Figure 2. 30.72 MHz ^2H NMR spectra of anabasine in $CHCl_3$ with natural abundance C^2HCl_3 as internal reference at δ 7.25: (*a*), anabasine [(**11**) and (**12**)] derived from (*S*)-[1-^{2}H]cadaverine; (*b*), anabasine [(**13**)–(**16**)] derived from (*R*)-[1-^{2}H]cadaverine.

[1-^{2}H]cadaverine is shown in Figure 2(*a*). Two signals with equal enrichment were observed for the axial and equatorial protons at C-6′ at δ 2.76 and 3.15. This labelling pattern indicates that the *pro-S* hydrogen is lost on oxidation of the primary amino group, probably by a diamine oxidase, and that equal amounts of (*R*)- and (*S*)-anabasine are produced. The bulky pyridine substituent takes up the preferred equatorial position giving rise to diastereoisomers of anabasine labelled with ^{2}H at 6′-H_{ax} (**11**) and 6′-H_{eq} (**12**). (Unlabelled material would be racemic.)



Ar = 3 - pyridyl

After feeding (*R*)-[1-^{2}H]cadaverine to *N. rustica* transformed root cultures, the ^{2}H NMR spectrum of the purified anabasine taken in chloroform showed three signals in the ratio 2:1:1 at δ 3.63, 3.21, and 2.81, respectively [Figure 2(*b*)]. This corresponds to ^{2}H present at C-2′, and C-6′ (equatorial and axial). It is clear that the *pro-R* hydrogen is retained on oxidation of the primary amino group leading to the retention of deuterium at C-2′. In addition, equal amounts of (*R*)- and (*S*)-anabasine are again formed, as shown by the appearance of ^{2}H at both axial and equatorial positions of C-6′. The labelling pattern for anabasine is consistent with the presence of equal amounts of four ^{2}H-labelled species (**13**)–(**16**). Species (**13**) and (**14**) constitute



Ar = 3-pyridyl

racemic 2′-^{2}H]anabasine, whereas species (**15**) and (**16**) are diastereomeric [6′-^{2}H]-forms of (*R*)- and (*S*)-anabasine. The ^{1}H NMR spectra of anabasine obtained after feeding (*R*)- and (*S*)-[1-^{2}H]cadaverine were difficult to interpret because of the mixture of labelled species present and some impurities in the small amount of material (*ca.* 1 mg) present. Confirmation of the presence of ^{2}H in the anabasine samples was obtained by mass spectrometry. After the root cultures were fed with (*S*)-[1-^{2}H]cadaverine, anabasine had peaks of roughly equal intensity at *m/z* 163 and 162 consistent with the presence of $^2H_1$ species in *ca.* 50% of molecules. On the other hand, after the root cultures were fed with (*R*)-[1-^{2}H]cadaverine, the anabasine sample showed a major peak at *m/z* 163 corresponding t› a high percentage of $^2H_1$ species.

In summary, it has been established, first, that only the piperidine ring of anabasine (**6**) is formed from the cadaverine (**2**) supplied to both *Nicotiana* cultures; secondly, that at least in *N. rustica*, only the *pro-S* hydrogen is lost on oxidation of one of the primary amino groups of cadaverine; and, finally, that (again in *N. rustica*) condensation of 2,3,4,5-tetrahydropyridine (**4**) with the nicotinic acid-derived moiety proceeds without stereoselectivity. (Racemisation of the anabasine is unlikely to occur after it is formed, because no loss of deuterium from C-2′ relative to C-6′ of the alkaloid could be detected after feeding with (*R*)-[1-^{2}H]cadaverine.) There is no evidence that the anabasine produced from cadaverine supplied to these cultures arises by a mechanism different from that operating in intact *Nicotiana* plants; in particular, the mechanism demonstrated *in vitro* by Hasse and Berg and by Mothes *et al.*[10] is not responsible. It is likely that the initial conversion of cadaverine into 5-aminopentanal (**3**) is catalysed by *N*-methylputrescine oxidase, which is active with this substrate despite a high apparent K_M value (2.75mM);[16] the subsequent chemistry may be non-enzymic, however. Leete[17] commented that, in contrast to nicotine, anabasine isolated from *N. glauca* was optically inactive and it seems probable that the final stages of anabasine and nicotine biosynthesis differ substantially; Wigle *et al.* have shown from feeding experiments with (*R*)- and (*S*)-[1-^{2}H]putrescine that only (*S*)-nicotine is formed in *N-tabacum*.[18] Finally, feeding (*R*)- and (*S*)-[1-^{2}H]cadaverine to plants or cell cultures of other genera which produce anabasine, in conjunction with ^{2}H NMR spectroscopy, would also be revealing and could be used to determine the optical purity of the anabasine, even when (as in this case) the amounts of anabasine isolated are too small to make reliable measurements of optical rotations.

Experimental

NMR spectra were recorded on a Bruker WP-200SY spectrometer operating at 30.72 MHz for ^{2}H, and on a JEOL

GX 400 spectrometer operating at 399.65 MHz for ^1H and 100.4 MHz for ^{13}C.

Cultures.—Hairy root cultures of *Nicotiana rustica* and *N. tabacum* transformed with *Agrobacterium rhizogenes* were established as described.[7,8] They were grown on Gamborg's B5 basal medium to which was added 80mM sucrose. At subculture, every 2 weeks, *ca.* 0.1 g roots was passaged into 50 ml medium in a 250 ml Erlenmeyer flask. Aqueous solutions of the labelled cadaverine dihydrochlorides (*ca.* 100 mg) were adjusted to pH 6, filter sterilised, and added to 10 flasks 6 d after sub-culture. Cultures were grown at 25 °C in normal laboratory light with shaking at 90 r.p.m. for a further 12 d. Weight of roots harvested was 50–100 g.

Precursors.—[2,2,4,4-$^2H_4$]Cadaverine dihydrochloride with a $^2H_4$ content of *ca.* 94% (^1H NMR spectrum) was prepared as described.[13] Samples of (*R*)- and (*S*)-[1-^2H]cadaverine dihydrochloride had $^2H_1$ contents of *ca.* 90% and 85% respectively.[14]

Isolation of Anabasine (**6**).—The roots were blended in 0.2% H_2SO_4 (100 ml), left for 2 h and filtered. The filtrate was washed with ether (100 ml), and then basified with 1M NaOH, and the solution was extracted with chloroform (4 × 100 ml). The chloroform extracts were dried, filtered, and concentrated to give a mixture of alkaloids (20–40 mg). Similar extraction of the basified culture medium produced very little additional alkaloidal material. GLC analysis of the alkaloid mixture on a silica capillary column coated with Durabond 1, and comparison with standards showed three major peaks with retention times 23.3 min (nicotine), 27.1 min (anabasine) and 27.7 min (anatabine). The alkaloids were also identified by HPLC–MS on a μBondapack C_{18} reverse phase column with a thermospray interface to a Kratos 50 mass spectrometer as described previously.[8] The alkaloids were separated by preparative TLC on silica gel plates (0.25 mm thickness) developed with chloroform–methanol (5:1). Anabasine, R_F 0.34, was removed and rechromatographed on silica gel plates (0.25 mm thickness) and eluted with toluene–acetone–methanol–25% aq. ammonia (8:9:2:1). Anabasine was identified by comparison with authentic material and detected by UV absorption and the modified Dragendorff reagent.[19] R_F Values (second solvent system): nicotine (**7**) 0.66, anabasine (**6**) 0.44, anatabine (**9**) 0.37, nornicotine (**8**) 0.31.

Unlabelled anabasine (Sigma) (**6**) (Found: M^+, 162.1152. Calc. for $C_{10}H_{14}N_2$: *M*, 162.1157); δ_C(100 MHz) and δ_H(400 MHz) data are given in the Table. ^2H NMR spectra were recorded at 30.72 MHz in $CHCl_3$ with natural abundance $CDCl_3$ as internal reference at δ 7.25. Anabasine (**10**) after feeding with [2,2,4,4-$^2H_4$]cadaverine dihydrochloride; δ_D 1.5–1.8, δ_H(400 MHz in $CDCl_3$) 1.45 and 1.86 (2 H + impurities, AB system, *J* 13 Hz, 4′-H_2), 2.76 and 3.15 (2 H, AB system, *J* 12 Hz, 6′-H_2), 3.62 (1 H, s, 2′-H), 7.2 (1 H, m, 5-H), 7.71 (1 H, d, *J* 8 Hz, 4-H), 8.43 (1 H, br s, 6-H), and 8.55 (1 H, br s, 2-H).

The ^2H NMR spectrum for anabasine (**11**) and (**12**) after feeding with (*S*)-[1-^2H]cadaverine dihydrochloride is shown in Figure 2(*a*), and that for anabasine (**13**)–(**16**) after feeding with (*R*)-[1-^2H]cadaverine dihydrochloride is shown in Figure 2(*b*).

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